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**Microbiological Characterisation of White Pigment Slurries**  
-  
**A Strategy for Bacteria Management**

**By**

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**Thesis Submitted for the Degree of Doctor of Philosophy**

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## **Author's Declaration**

The work contained in this thesis is the result of original work by myself under the supervision of Dr. C. S. Dow, unless otherwise stated. All sources of information have been acknowledged by means of reference. None of the work contained in this thesis has been submitted for any previous degree.

This study was financed by Omya AG in Switzerland and carried out in collaboration with Omya's laboratories in Oftringen, Switzerland, under the local supervision of Dr. Patrick A.C. Gane.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

**Patrick Schwarzentruher**

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## **Publications**

1. Schwarzentruher, P., Gane, P.A.C., 2003. Application of Microbiocides for the Storage Protection of Mineral Dispersions. In Paulus, W., Directory of Microbicides for the Protection of Materials and Processes. Dordrecht: Kluwer Academic Publishers.
2. Schwarzentruher, P., 2002. CellFacts II - Single Cell Analyses in Real Time. In Adler, H.-J. P., Potje-Kamloth, K., Quo Vadis - Coatings. Weinheim: Wiley-VCH Verlag GmbH.
3. Schwarzentruher, P., 2002. Mikrobiologische Lack-Analyse in Echtzeit. Farbe & Lack, 108; pp. 24-26.
4. Schwarzentruher, P., 2003. A New Analytical Method in Microbiology. In Meine, D., Novel Biocide Technology. Hanover: VINCENTZ Verlag.

## **Presentations and Conferences Attended**

1. Münchner Papier Seminar - Mikrobielle Probleme in Munich, September 25<sup>th</sup>, 2000. Attended.
2. Schwarzentruher, P., CellFacts II - Eine neue Analysenmethode in der Mikrobiologie; Oral presentation held at the 1<sup>st</sup> Omya Paint Seminar in Cologne, February 20<sup>th</sup>, 2002.
3. Schwarzentruher, P., Microorganisms in White Pigment Slurries; Oral presentation held at the Asia Pacific Production Conference in Bangkok, April 30<sup>th</sup>-May 2<sup>nd</sup>, 2002.
4. X<sup>th</sup> International Congress of Bacteriology and Applied Microbiology in Paris, July 29<sup>th</sup>-August 1<sup>st</sup>, 2002. Attended.



5. Schwarzentruher, P., Bacteria Management; Oral presentation held at the Carbonate Day (Conference for Finnish Paper Industry) in Lappeenranta, August 28<sup>th</sup>, 2002.
6. Schwarzentruher, P., CellFacts II - Single Cell Analysis in Real Time; Oral presentation held at the XXVI FATIPEC Congress in Dresden, September 9<sup>th</sup>-11<sup>th</sup>, 2002.
7. Schwarzentruher, P., A New Analytical Method in Microbiology; Oral presentation held at the European Coating Conference in Berlin, February 6<sup>th</sup>-7<sup>th</sup>, 2003.
8. BioTrends - Life Science meets Nano, International Congress & Exhibition in Berlin, February 11<sup>th</sup>-14<sup>th</sup>, 2003.

## **Patents**

1. Schwarzentruher, P., Buri, M., 1999. Patent # DE 199 04 057. Process for determination of microbial contamination.
2. Schwarzentruher, P., Buri, M., 2001. Patent # 01943291.3-2103-EP0104729. Low-freezing point formulation containing phenol and/or phenol derivatives.

## **Membership of Professional Organisations**

- Since 2001, Member of the Society of Applied Microbiology
- Since 2001, Member of the Society of General Microbiology

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## Abbreviations

API	Appareil Procéder D'Identification
ATP	Adenosine Triphosphate
BIT	1,2-Benzisothiazoline-3-one
bp	Base Pairs
BPD	Biocidal Product Directive
BSA	Albumin from Bovine Serum
CMIT	5-Chloro-2-Mmethyl-4-Isothiazoline-3-one
DB	Disruption Buffer
DMSO	Dimethyl Sulfoxide
dmt	dry metric ton
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetraacetic Acid
EG	Ethylene Glycol
EGH	Ethylene Glycol Hemiformal ([1,2-ethanediylbis(oxy)]-bis-methanol)
FC	Flash Cooler
FISH	Fluorescent <i>in situ</i> Hybridisation
GCC	Ground Calcium Carbonate
MIC	Minimum Growth Inhibitory Concentration
MIT	2-Methyl-4-Isothiazoline-3-one
MW	Molecular Weight
NA	Nutrient Agar
NTP	Nucleosid Triphosphate
OMF	Omyafil
OPP	o-Phenylphenol
OPQ	Omyapaque
PAA	Polyacrylic Acid
PBS	Phosphate Buffered Saline
PCA	Plate Count Agar

PCC	Precipitated Calcium Carbonate
PCR	Polymerase Chain Reaction
PX	Final Grinding Machine
RC	Rail Car
RCW	Recirculation Water
RISA	rDNA Internal Spacer Area
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SRB	Sulphate Reducing Bacteria
TBE	Tris-borate/EDTA
TBS	Tris-buffered Saline
TTC	Triphenyl Tetrazolium Chloride
TVC	Total Viable Count
VDU	Visual Display Unit
WWD	Warm Water Dilution



## Summary

The microbiological characterisation as well as the application of microbiocides for the storage and protection of mineral dispersions is of ever-increasing interest for scientists and industrialists and includes many challenges for the mineral slurry producer and user. Increasing conversion from dry pigment handling to water-based dispersions is taking place over a wide range of production applications, for example, papermaking filler products and coating formulations in both the paper and paint industries.

The requirements for the delivery of preserved slurried products begins from the moment the mineral is extracted or synthetically produced. The process conditions are as important regarding bacterial colonisation and control as the delivery and storage strategy of the end-product itself. This thesis attempts to give a detailed insight into the background issues and procedures needed to provide an environment of "good housekeeping", essential in optimising the microbiological control needed for preservation and acceptable application of the pigment in its end-use. On this base, the latest research on the bacterial strains, their identification, measurement and growth dynamics in real-time are presented, and new biocide strategies, applicability and constraints are discussed. Illustrations are given throughout of the sources of microbiological contamination likely to occur during production, storage and transportation.

Based on the current knowledge being gained from combining active Research and Development and on the ground applications expertise, new possibilities for optimising microbiological quality control are described.

# **CHAPTER 1**

## **Introduction**

### 1.1 White Pigment Slurries

The use of minerals has become indispensable in everyday life. Many different stones and powdered minerals have already been used for thousands of years; also the White Pigments (calcium carbonate, clay and talc). They are used in the sculpture, the building and chemical industries, as fillers in paper, paints and plastics as well as in the pharmaceutical industry. Here, the calcium carbonate stones have especially to be mentioned. Their enormous economic importance becomes apparent if one looks at the quantities of chalk, limestone and marble that are utilised year by year. In 1994, for example, 4.6 billion tonnes of limestone were used worldwide (Tegethoff *et al.*, 2001).

The use of White Pigment Slurries as fillers and pigments has attained great importance in the paper industry. The excellent optical and mechanical properties, which influence the brightness, strength, glaze and porosity of the paper, lead to better printing and lettering properties of the paper (Figure 1). This trend is also increasingly noticeable in the paint industry. With a production of more than 12 million tonnes of White Pigment Slurries per year, Omya is one of the largest producers in the world. The production of the slurries at several production sites (Figure 2) all over the world guarantees the availability of the product at any time. To be able to deliver the enormous quantities of White Pigment Slurries to customers worldwide just in time presents huge logistical problems. Storage tanks with a capacity of up to 3500 m<sup>3</sup> as well as slurry tankers with up to 16,000 grt are indispensable (Figure 7).

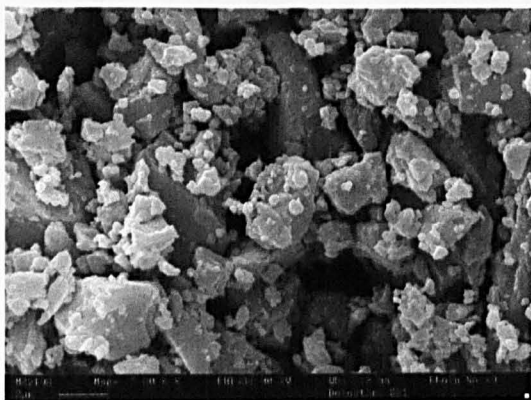


Figure 1. Scanning electron micrograph (magnification: 5000x) of a marble stone from Carrara (Italy). High brightness in paper as well as in paints are achieved with this quality of crushed marble.

These huge production and storage capacities make high demands on securing product quality. To be able to control microbial growth or eliminate microbes by the use of biocides considerable efforts with respect to hygiene in the whole production process are necessary.



Figure 2. The Omya calcium carbonate production site in Molde (Norway). The docked slurry tanker is being loaded directly from the slurry storage tanks of which there are 48 at the Molde site varying in capacity from 1500-3500 m<sup>3</sup>. The microbiological control of such large quantities of slurry requires enormous efforts in monitoring, preservation and hygiene.

Increasing restrictions and limitations on the use of biocides and the quantities involved complicates their use both in pigment supply as well as in its use in the paper and paint industries. To be able to guarantee the quality of the product in the area of microbiology, systems allowing monitoring in real time are required, in addition to environmentally friendly and efficient biocides and good house-keeping procedures.

### 1.1.1 Manufacturing Process

From the mining of raw stone to the delivery of a pigment suspension to the paper industry, the material passes through a wide range of processing steps and procedures, typically as might be seen schematically in Figure 1 (Tegethoff *et al.*, 2001). Following the breaking of the mineral or mineral-containing stone, the first crushing step, the material is usually washed and prepared for optical and size selective sorting. To separate unwanted impurities flotation is often carried out prior to further stringent size classification by hydrocyclone or centrifugation. If synthetic pigments are to be made, the raw material, such as limestone, prior to burning or calcining, must also be carefully selected. The slaking process for precipitated calcium carbonate, for example, requires specific control of the burnt lime source and particle sizing before the carbon dioxide addition stage.

Adjusting the parameters of wet (water-borne) fillers or pigments to the respective requirements of paper making and coating is achieved by control of crystal growth, in the case of precipitated products such as precipitated calcium carbonate or precipitated silica, control of particle delamination and comminution, in the cases of kaolin, mica and talc, or by grinding and selection technology in a wet milling process, for materials such as ground calcium carbonate. Each methodology is pursued at a controlled solids content in either a dispersed or a flocculated state, depending on the mineral base and the use of various dispersants and flocculants. Final product dispersions depend on the concentration of the chosen particle size and shape distributions, their state of dispersion and the intrinsic particle-particle packing characteristics. When transportation over long distances is required, methods to achieve the maximum solids content within the constraints of the final application are strenuously sought.

During the fine grinding processes, for example, temperatures of up to 110°C can be reached. This ensures, to a large extent, the thermal disinfection of the processed mineral. Thus, there is generally a significant reduction in the bacterial count, arising initially from contaminated plant and treatment waters and process additives, from that found in the feed material. After such a grinding or high temperature processing step, the dispersions can often be regarded in microbiological terms as of pharmaceutical quality. There are, however, some cells which, due to certain



protection mechanisms, are not destroyed by the grinding process but are transferred into a physiologically stressed or dormant state. During slurry product storage, post-dispersion of synthetic products, or during transportation, the temperature can decrease (or rise again) to a level which is favourable to bacterial growth (25 - 45°C). This growth then proceeds once either infection occurs from outside sources, usually by fluid contamination or even contact with air, or by the further viability of the dormant or stressed cells. It is this post-processing contamination that focuses most attention when considering slurry preservation. Of course, should a processing step involve lower temperature handling or combinations of products, then a complete revision of the process environment in respect to bacterial contamination must be made. This is specific to the plant in question and requires extensive analysis of water systems, settling and waste recovery vessels, and air-borne sources, such as air-conditioning and ventilation.

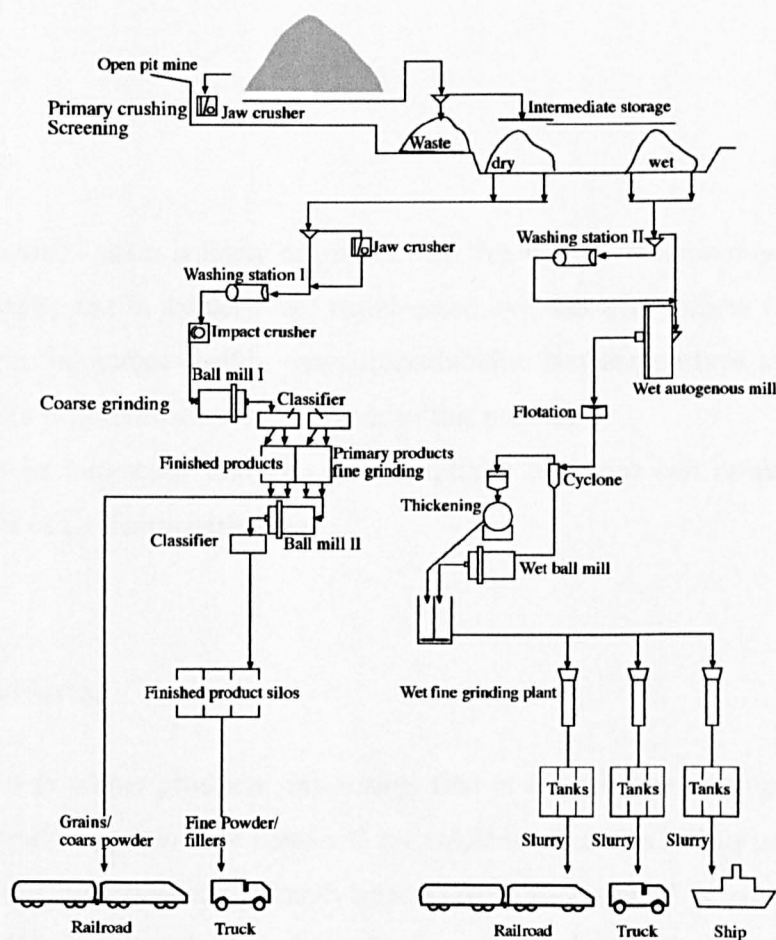


Figure 3. Schematic manufacturing process of dry and wet White Pigment product.

### 1.1.2 Range of Products

The use of minerals as fillers or pigments has become indispensable for everyday life. The main function of a White Pigment Slurry is to improve the technical properties of products like paper, plastics or paints and lacquers. For this purpose, minerals like kaolin, talc and calcium carbonate are particularly suitable; but titanium dioxide, magnesium carbonate, barium and calcium sulfate also have interesting properties (Tegethoff *et al.*, 2001).

The mineral calcium carbonate occurs in different forms, all of them being suitable as fillers or pigments, namely chalk, limestone and marble. In addition, there is the synthetically produced, precipitated calcium carbonate, the PCC (Precipitated Calcium Carbonate). Although all of these forms are chemically identical, each of them has specific features that make it different from the others.

#### 1.1.2.1 Limestone

Limestone in its natural form is finely dispersed. For this reason, limestone was the first calcium carbonate filler which was in former times mainly used in paints and putties. The quality of being recognised as safe for human health, easy processability, lamellar particle structure as well as good coatability are properties arguing in favour of this mineral.

However, due to its biogenesis and low metamorphosis limestone can never reach the highest possible brightness of a calcium carbonate.

#### 1.1.2.2 Chalk and Marble

As the desire for ever whiter products was arising, first in the paint and lacquer industry and later also in the paper industry, new base materials for calcium carbonate had to be sought; limestone could not fulfil this requirement. Through appropriate processing it is possible to obtain the desired high brightness values both with chalk as well as with marble. The highest brightness

values are however achieved with marble.

Chalk and marble do not only differ from limestone in their brightness, the particle size distribution, abrasiveness and opacity of the fillers also vary.

#### 1.1.2.3 PCC

A further form of calcium carbonate is PCC, the precipitated calcium carbonate. The appreciation of this synthetically produced calcium carbonate, for example in the paper industry, is mainly due to the fact that freshly precipitated PCC has a higher volume than natural calcium carbonates (GCC). The metamorphosis of marble can, however, not be completely reproduced in the laboratory.

#### 1.1.2.4 Clay

Before calcium carbonate was used as a filler and/or pigment in the paper industry, clay was one of the most important and most used minerals. Today, clay is used in combination with calcium carbonate and talc for the production of high-quality printing paper. In North America, clay is still the most frequently used pigment in the paper industry, although it has to be mentioned that the development of "new" paper minerals was started there with a delay of about 15 years.

Kaolin (kaolinite), so-called after the first known use for pottery manufacture by the Chinese of white alumino-silicates found in the Kao-Ling district, is a 1:1 layer silicate and has for many years been the major pigment form used in acid papermaking to add smoothness and opacity to paper. It has an idealised structural formula of  $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$  (Gane, 2001).



#### 1.1.2.5 Talc

Talc is another frequently used platy pigment structure and is an example of a 2:1 layer silicate with an idealised structural formula expressed as  $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$ . The layers of talc are electrostatically neutral and are held together via van der Waals bonding. As a result of neutrality, talc is strongly oleophilic and therefore its hydrophobicity creates challenges in dispersability in aqueous systems. However, the surface properties are very valuable in respect of adsorption of sticky and other aliphatic compounds for pitch control in papermaking and attractiveness toward oils, toluol and esters make it a useful additive in coatings for control of offset and rotogravure printability (Gane, 2001).

#### 1.1.3 Production Application

Marble products are today the favourite fillers and coating pigments in the paper industry. However, because of their high brightness they are also interesting for the paints and lacquer industry since they help to reduce the content of expensive white pigments such as titanium dioxide in colours.

The fields of application of marble fillers are similar to those of PCC which, due to its purity, is also used in pharmaceutical products.

Chalk products are mainly used in fields where the contribution to the added value of the finished paper is comparatively small and therefore raw materials with lower brightness can be used without loss in quality. For flue-gas desulfurisation or fertilisation of forests, therefore, mainly chalk is applied. However, there also exist superior chalk qualities which are used as fillers or pigments in the paper industry as well as in the paints and lacquer industries.

The fillers made from limestone cannot be clearly assigned to a defined application. There exist high-quality limestones which, due to their positive properties, are still used in the paper, paints and lacquer industries as well as for the production of cables. There are also the other, not so pure, limestones which serve as raw materials for the production of fertilisers and similar fields of application.

#### 1.1.4 Organic Additives for Dispersion Stabilisation

To be able to produce suspensions having controlled solids contents, of more than 70% (w/w), special dispersants are required. Without such dispersants, a mixture containing a mineral, such as calcium carbonate, with only 30% (w/w) water is no longer flowable. Pigment and filler producers have developed highly active polymer systems, especially for the use of mineral slurries in the paper industry, which allow such concentrations to be achieved without destroying the complex chemistry of a paper machine.

The dispersants, today are usually based on salts of polyacrylic acid (PAA), and represent a rich supply of organic nutrients for microbes. They also serve as both a carbon and an energy source. Mineral dispersions, however, also contain a series of other important biologically supportive substrates which contain oxygen, nitrogen, calcium, magnesium, sodium, potassium, phosphorus, sulfur and iron, all of which are essential for energy metabolism (Schwarzentruber & Gane, 2003).

Microbial growth in a mineral dispersion can be influenced by many different factors, such as:

- Higher solids content of a dispersion makes growth beyond a certain concentration more difficult as the physical space for microorganisms is reduced. As a result, dispersions with high solids contents are often easier to preserve.
- Higher salt concentrations lead to a differential osmotic pressure across the microbial cell envelope. The effects are selective to the species present in a system. Usually it is a change in salt concentration, rather than an absolute level, that can have an inhibiting effect on the growth of microorganisms already existing in the system.

### 1.1.5 Microbial Contamination and its Consequences

Microorganisms are omnipresent on earth (and maybe beyond) and, of course, mineral dispersions are no exception. Bacterial counts of  $> 10^6$  cfu ml<sup>-1</sup> can lead to unpleasant odour, discoloration, acidification and viscous build-up. Under certain conditions of aeration, followed by stagnation, strong initial aerobic growth, eventually consuming the oxygen present, can subsequently create the conditions for anaerobic growth, which is generally connected with a decrease in the redox potential. Furthermore, a decrease in pH, and the often associated increase in viscosity, can lead to considerable problems for the final user. The rheological properties of mineral dispersions are extremely important for the processing of the product (e.g. pumpability, filtering, rheological flow characteristics in a coating head and in recirculation systems).

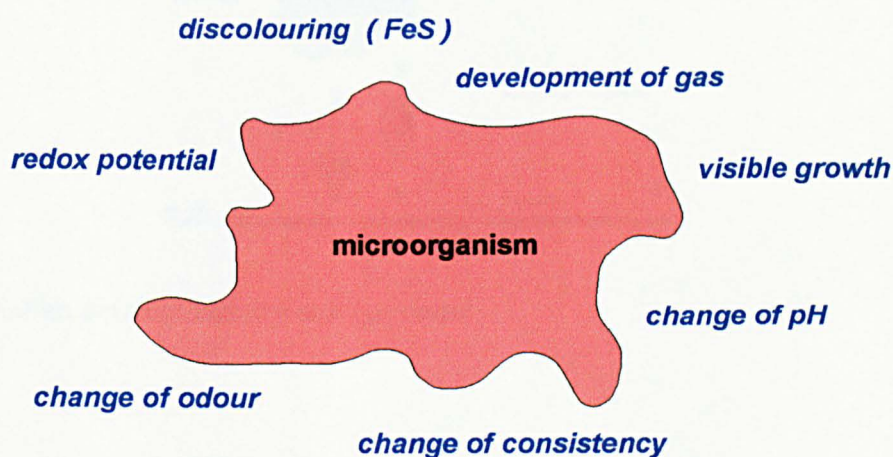


Figure 4. Consequences of microbial contamination in White Pigment Slurries.

Furthermore, there is a risk of uncontrolled deposition (biofilms), which, for example in paper production, can lead to holes and breaks in the paper web.

Clearly, the need for biocide(s) to initiate, preserve and maintain slurry purity is a very important part of the slurry producer's and handler's requirements for efficient application and storage of minerals.

Biocide in the slurry itself, however, is only one aspect of preservation. In order to keep mineral slurries clean in storage tanks and during transportation (truck, rail waggon, boat) (Figure 7), and to achieve good performance of biocide(s) within the product, the effect of headspace preservation as well as the cleaning of pipes and transportation facilities must not be underestimated (Figure 8). Contamination from the vessel headspace can be a major source of biofilm development which has a high potential for recontamination of mineral slurries (Figure 5 & 6). Biofilms can also occur in places where mechanical cleaning is difficult, i.e. dead legs in pipework and storage or reaction vessels. In these cases, where biofilms flourish, there is often a limit to the practical chemical disinfection and preservation that can be attained, due to the impermeability of many of these types of films. The need to avoid stagnation in plant design is, therefore, paramount.

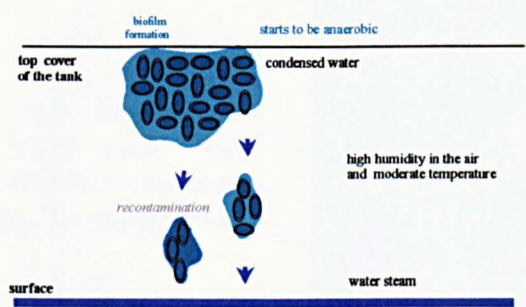


Figure 5. Biofilm development in a storage vessel.

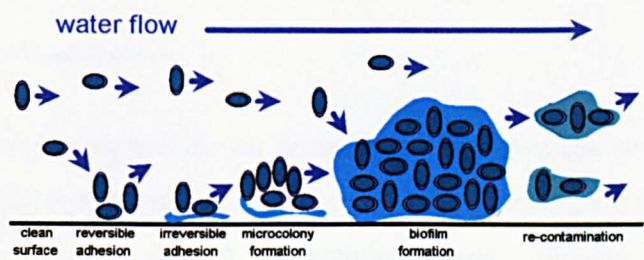


Figure 6. Biofilm development in pipework.



The target, therefore, is not only microbial control within the product itself but also the control within the confines of the immediate product-contact environment.



Figure 7. To meet today's logistic requirements, slurry tank vessels with a capacity of up to 16,000 registered tonnes are in operation.

The transport of these large quantities of mineral slurry dispersions makes great demands upon preservation and brings a new meaning to being "ship-shape".

Figure 8. Emptied rail tank waggons are cleaned with fresh water under high pressure to guarantee optimum conditions with respect to cleanliness for the next load to be transported.



1.1.6 The Need for Preservatives

Biocides have become indispensable for ensuring the high hygiene and quality standard of White Pigment Slurries. Microorganisms - such as bacteria, for example, are omnipresent. They endanger the rheological as well as optical characteristics and products become unusable due to contamination. Remedial action is primarily taken by using chemical substances with which the organisms can be combated. The definitive action of biocides is to act selectively against organisms.

Preservatives (biocides) are defined chemical substances or mixtures of substances having a low

toxicity and good skin tolerance. Low concentrations, usually in the range of 100 - 1000 ppm per dmt White Pigment, destroy microorganisms or inhibit their development while showing good compatibility with the product they are used to protect and ensure clean storage as well as clean transportation to the customer.

The products in use today are mature and reliable with respect to their effectiveness.

New active agents are rarely found these days, apart from modifications of known substances.

## 1.2 Status of Knowledge

### 1.2.1 Previous Work

There are a few published scientific studies on the microbiology of White Pigment Slurries and their preservation and/or disinfection, as a result of which little is known of the microbial diversity in this environment.

Traditionally, the determination of a TVC was done by plating White Pigment Slurries on agar that reflected to some extent the properties of the medium that they came from e.g. dispersants have a high salt content, and its pH can support the growth of fungi, whereas slurries have a pH between 7 and 10 and especially calcium carbonate slurries are too alkaline for the growth of yeast and fungi. In addition to this, analysis of complex microbial consortia has rarely been done and relied on culture methods using a variety of media designed to maximise the recovery of different microbial species. Initial microbiological analysis of a range of White Pigment Slurries employing traditional plating techniques typically revealed the presence of only one or two bacterial colony types which were mostly determined as pseudomonads.

As a result of this, studies of preservatives/disinfectants were often carried out using test microbes that are frequently used as standards for the determination of the bactericidal action of antibiotics, but do not occur in White Pigment Slurries due to the pH or nutrient content. This often led to misinterpretation of the effectiveness of bactericidal substances intended for use as preservatives for slurried products.

### 1.2.2 Existing Technologies - Advantages / Disadvantages

The restrictions and potential biases of established techniques such as plating have been frequently reported, and it may be that for many environmental samples the majority of microscopically visualised cells are viable but do not form visible colonies on plates - the "great plate anomaly". There are two different types of cell that contribute to this silent but active majority: (a) known species for which the applied cultivation conditions are not suitable or which have entered a non-culturable state such as compromised cells which recover if i.e. microbiocidal stress is removed or dormant cells, (b) unknown species that have never been cultured before due to the lack of suitable methods. Both of these possibilities may apply to White Pigment Slurries. Indeed, it has been estimated that less than 0.1% of the microorganisms found in typical agricultural soils are culturable using current media formulations (based on comparisons between direct microscopic counts and recoverable cfu's) (Amann *et al.*, 1995). Whilst methods based on physiological profiles of isolates such as API testing may provide useful information, they are still subject to the same bias problems encountered with culture plating methods, making data interpretation problematic, and leading to false results.

Despite the fact that culture-dependent techniques are not ideal for studies of the composition of natural microbial communities when used alone, they provide one of the more useful means of understanding the growth habits, development, interactions and potential function of microorganisms from White Pigment Slurries. As such, a combination of culture-based and culture-independent approaches is likely to reveal more complete information regarding the composition of White Pigment Slurry communities.

The ATP method is easily and rapidly applicable, though it is not possible to determine the total viable count but just a trend. White Pigment Slurries, however, are a poor medium for the measurement of ATP from bacterial cells. The turbidity masks/adsorbs the light emission from an ATP assay, thus leading to significant measuring errors. To be able to avoid these effects the sample would have to be so strongly diluted that the cell number would be below the measurable range. Furthermore, ions, pH, temperature, chemicals as well as enzymes can influence the measurement.

A further method which is partly used in the paper industry for the determination of the bacterial count is the measurement of the RedOx potential. However, like with the ATP method, it is not possible to determine bacterial counts but only trends. It has however to be remembered here that even a statement about a trend has to be used with the utmost caution. A strongly negative RedOx potential is taken to be indicative of strong growth of microorganisms. A strongly negative RedOx potential is however only caused by SRBs and a few other anaerobes. Nevertheless, evidence of the presence of SRBs often comes too late since strong aerobic contamination has already occurred, which has a significant impact on the physical properties of White Pigment Slurries. Furthermore, it has to be remembered that, both the pigment as well as the paper industry, also use preservatives which themselves show a strongly negative RedOx potential.

### 1.3 Information Required by Omya

The increasing sensitivity of the paper as well as the paint and lacquer industries with respect to the microbiological "purity" of White Pigment Slurries compelled the pigment industry into enormous investment in the areas of preservation, house-keeping and microbiological quality control. The parameters described in this paragraph are the main points to be considered in order to fulfil the high demands of the users of White Pigment Slurries.

#### 1.3.1 Sources of Contamination

A first important step for a specific and effective control of microbiological activities in a White Pigment Slurry is knowledge of the potential sources of contamination. Once potential sources of contamination are known appropriate measures can be taken to eliminate or prevent them in future. This guarantees optimisation of the action of biocides and, at the same time, leads to a reduction of costs.



### 1.3.2 Total Viable Count

The determination of the Total Viable Count currently serves as the microbiological quality control parameter for the products. In most cases, but mainly in Europe, the Total Viable Count in White Pigment Slurries should not exceed 1000 cfu ml<sup>-1</sup>. In the paper, paint and lacquer industries, samples are often drawn on arrival of the products and analysed for their Total Viable Count. If this is beyond the agreed specification (e.g. > 1000 cfu ml<sup>-1</sup>) the customer reserves the right to complain about the product or, in the worst case, to reject it. The determination of the Total Viable Count also gives information on where contamination has occurred and how well the slurry has been stabilised by biocides.

### 1.3.3 Microbial Characterisation

In the past, the pigment industry as well as their customers were not interested in knowing which species can occur or be detected in White Pigment Slurries. This attitude however changed when CaCO<sub>3</sub> was also supplied to the food industry and bacteria increasingly formed resistance to the biocidal agents used.

In the present study the implementation of molecular-biological methods such as PCR, FISH, RISA and DNA probes has shown the variety of different types of bacteria in White Pigment Slurries. For years, this had been reduced to the presence of pseudomonad species.

The knowledge of which species are present in a product or on a production site makes it possible to modify and adjust bactericidal agents and to meet the high requirements of the food industry concerning microbiological purity.

### 1.3.4 Microbial Physiology

The information about the physiological state of a bacterial cell is extremely important for the determination of the optimum time for the addition of preservatives, on the one hand, and for being able to check their effectiveness, on the other hand.

With this information it is possible to test the speed of the bactericidal action of a preservative on a spectrum of microbes and, at the same time, to determine whether all bacteria can be eradicated by this active agent.

The importance of the addition of the preservative to achieve an optimum effect, e.g. at the initial stage of the growth phase of the microorganisms, should not be underestimated.

#### 1.3.4.1 Viable / Dead

Molecular-biological methods such as DNA probes do not supply any information about the viability of cells. Fluorescence techniques open many more possibilities. Due to the membrane potential of a cell it is possible to determine whether a cell is viable or dead (Haughland, 1996).

A further advantage of this technique is the possibility to obtain such information *in situ* and in real time.

#### 1.3.4.2 Stress / Dormancy

Despite the high temperatures of up to 110°C which are reached during fine grinding (section 1.1.1) sterility is not achieved. Investigations have shown that the majority of cells are destroyed during the grinding process, while some are just put into a "stressed state" and survive (section 3.3.2). If the White Pigment Slurry cools down during storage or transport the stressed cells can recover and thus show "viability". The determination of stressed or dormant cells is of considerable interest since these cells can use up the preservative and prepare the environment for further growth by additional recontamination. The fact that stressed or dormant cells cannot be detected using traditional techniques such as the plate count highlights the need of a technology for the detection of such cells.

### 1.3.5 Effectiveness of Preservatives / Disinfectants

The spectrum of activities as well as the speed of action of a preservative/disinfectant is extremely important for the quality of a White Pigment Slurry. To be able to guarantee that the bacterial count on arrival of the product at the customer's site is  $< 1000 \text{ cfu ml}^{-1}$  it has to be made sure that the active agents eliminate all microorganisms that are present in a system. The speed of the bactericidal action of preservatives/disinfectants should not be underestimated either. If contamination in a storage tank is detected, the bactericidal action in the case of adding additional biocide to the product should be apparent within a few hours or, preferably, within a few minutes. A fast reaction to contamination guarantees that the White Pigment Slurry is clean when it is loaded onto a ship or a rail waggon. The retention of a product in a storage tank for several hours (even days) to allow the biocide to act or whilst awaiting confirmation of its effect involves high costs and this should not be underestimated.

### 1.3.6 Stability of Preservation

The stability of preservation is especially important where long transport times to the customer exist. In North America, White Pigment Slurries are delivered from the East Coast to the West Coast and in Europe, customers located in southern Europe are supplied with products from Norway which are transferred via transit tank farms in Holland or Germany.

The stability can be influenced by different factors. The temperature during the addition of preservatives / disinfectants, for example, is an important factor. Most active agents are not, or only partially, temperature-stable, i.e. most of them are thermally decomposed at temperatures above  $50^{\circ}\text{C}$ . In addition, chemical cross-reactions can occur. Oxidising and reducing agents, amines, acids or alkali are just some examples of substances which can impair the stability of bactericidal substances. Prior to the use of a preservative / disinfectant it is therefore important to check with which substances incompatibility could be expected.

### 1.3.7 Environmental Impact

There are two essential points which have to be discussed here. On the one hand, the preservatives used by the producer of White Pigment Slurries must not have a negative effect on the efficiency of a water treatment plant in the paper industry and, on the other hand, releases of e.g. formaldehyde caused by formaldehyde-releasing preservatives have to be minimised as, otherwise, the customer runs the risk of not meeting with the Clear Air Act of their country or district.

For producers of White Pigment Slurry it is therefore important to know which impact the respective bactericidal agents and/or their decomposition products can have on the environment (i.e. water, air and soil).

### 1.3.8 Cost Reduction by Optimisation

The information given in sections 1.3.1 - 1.3.7, enables both optimisation of the production and preservation process as well as a reduction of the total cost of the microbiological stabilisation of White Pigment Slurries.

Fast methods for the determination of the Total Viable Count allow a quick reaction, i.e. early microbiological activity can be stopped or eliminated. Furthermore, the information about the sources of contamination and the species present enables selective use of preservatives. Increased stability of the product will lead to a direct reduction of costs as has been confirmed in production (section 3.7).

## 1.4 Aims of the Project

The aims of this work were:

- To characterise and identify the variety of microorganisms present in White Pigment Slurries.
- With this information to investigate new, fast measuring methods in the field of microbiological analysis and their application in production for quality control.
- To exploit fluorescence techniques which supply information on the physiological state of individual cells. This should result in a better understanding of the mechanism of action of preservatives / disinfectants.
- To optimise the use of preservatives / disinfectants with respect to house-keeping at production sites.

The synergy of these four main topics should result in advantages with respect to cost optimisation, an important factor which has clearly to be considered in industrial projects.

## **CHAPTER 2**

### **Materials and Methods**

## 2.1 Materials

Inorganic chemicals and solvents were mainly obtained from Sigma Aldrige Chemie GmbH (91625 Schnelldorf, Germany), Fluka Chemie AG (9470 Buchs, Switzerland), or Merck KGaA (64271 Darmstadt, Germany).

Anaerobic agar, Beads Working Solution, Columbia Agar, (DiSC<sub>3</sub>(5)), Disruption Buffer, Malt Extract agar, Nutrient broth, Nycodenz, PBS, Plate Count agar, Sabouraud-4%-Glucose agar, Sporulation medium, Syto 62, TBS, Tryptone, Tryptic Soy agar, Tryptic Soy broth were obtained from Difco Laboratories (Sparks, Maryland 21152, USA), bioMérieux Suisse s.a. (1202 Geneva, Switzerland) or Merck KGaA (64271 Darmstadt, Germany).

The majority of biochemicals were from Sigma Aldrige Chemie GmbH (91625 Schnelldorf, Germany), Molecular Probes (2333 AA Leiden, The Netherlands), Promega Corporation (Madison, Wisconsin 53711, USA) or QIAGEN AG (4052 Basel, Switzerland).

Unless otherwise stated, the water used was distilled water.

### 2.1.1 White Pigment Slurries

Calcium carbonate slurries from different Omya production sites in Europe, North America, Australia and Asia were used. These were aqueous dispersions of natural, ground marble, limestone or chalk having a solids content of 60 - 80% (w/w) and a particle size of 50 - 99% < 2 µm, dispersed using 0.2 - 1.3% (w/w) of a commercially available sodium polyacrylate.

Clay slurries from a production site in North America were used. These were aqueous dispersions of ground clay having a solids content of 60 - 75% (w/w) and a particle shape from rocky to totally delaminated with a size of 30 - 99% < 2µm, dispersed using 0.03 - 0.35% w/w of a commercially available sodium polyacrylate or sodium polyphosphate or blends thereof.

Talc slurries from different Omya production sites in Europe and Australia were used. These were aqueous dispersions of ground talc having a solids content of 40 - 65% (w/w) and a particle size of 20 - 70% < 2µm, dispersed with 0.3 - 1.2% (w/w) of a commercially available wetting agent (ethylene propylene oxide or lignin sulphonate) or with 0.05 - 0.3% (w/w) of a commercially available sodium polyacrylate.

### 2.1.2 Preservatives / Disinfectants

The preservatives and disinfectants used for the study are shown in Table 1:

Preservatives			
Trade Name	Supplier	Active Ingredients	% Active
Biocide A	Bode Chemie	[1,2-ethanediylbis(oxy)]-bis-methanol	92
Biocide AK	Bode Chemie	[1,2-ethanediylbis(oxy)]-bis-methanol 2-methyl-4- isothiazoline-3-one 5-chloro-2-methyl-4-isothiazoline-3-one	78 0.25 0.75
Biocide ABK	Bode Chemie	[1,2-ethanediylbis(oxy)]-bis-methanol 2-bromo-2-nitropropane-1,3-diol 2-methyl-4- isothiazoline-3-one 5-chloro-2-methyl-4-isothiazoline-3-one	68 10 0.25 0.75
Biocide BK14	Bode Chemie	2-bromo-2-nitropropane-1,3-diol 2-methyl-4- isothiazoline-3-one 5-chloro-2-methyl-4-isothiazoline-3-one	14 0.35 1.05
Ebotec BT20	Bode Chemie	1,2-benzisothiazoline-3-one	20
AMA 420	Vinings	3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione	20
Preventol ON 20	Bayer AG	o-phenylphenol	20
Preventol OF 45	Bayer AG	o-phenylphenol	45
Dowicil QK-20	DOW	2,2-dibromo-3-nitrilopropionamide	20
Dowicil 75	DOW	1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride	67.50
-	Merck KGaA	glutaraldehyde	50



Disinfectants			
Trade Name	Manufacturer	Active Ingredients	% Active
Tolcide PS75	Rhodia	tetrakis(hydroxymethyl) phosphonium sulphate	75
-	Merck KGaA	hydrogen peroxide	35
N-909	Verichem	sodium hypochlorite	12.5

Table 1. Preservatives and disinfectants used for the study.

### 2.1.3 Bacterial Reference Strains and Plasmids

Bacterial reference strains were from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (38124 Braunschweig, Germany) or isolated from White Pigment Slurries. The strains used in this study are described in Table 2.

<i>Bacillus subtilis</i>			
Strain	Information	Source	Reference
347	Produces restriction endonuclease <i>Bsu</i> 6633. Resistant to nisin. Peptide antibiotics. Production of bacilysin and fengymycin. Quality control strain according to DIN 58959-7 and European Pharmacopoeia, assay of antibiotics, test strain for growth media, sterility testing. Test for antimicrobial compounds in paper, cardboard, etc.	DSMZ	Lovett & Young, 1969
<i>Bacillus cereus</i>			
Strain	Information	Source	Reference
31	Type strain. Taxonomy / description. Produces restriction endonuclease <i>Bce</i> 14579. Nutrition.	DSMZ	Skerman, McGowan & Sneath, 1980
<i>Geobacillus stearothermophilus</i>			
Strain	Information	Source	Reference
22	Produces restriction endonuclease <i>Bst</i> PI ( <i>Bst</i> EII), phosphinothricin. Growth requirements. Sterilisation control.	DSMZ	Fahmy, Flossdorf & Claus, 1985

<i>Pseudomonas aeruginosa</i>			
Strain	Information	Source	Reference
50071	Type strain (1300). API 20 E, quality control strain.	DSMZ	Skerman, McGowan & Sneath, 1980
<i>Pseudomonas pseudoalcaligenes</i>			
Strain	Information	Source	Reference
7521	BIOLOG GN Micro Plate, quality control strain	DSMZ	Stanier, Palleroni & Doudoroff, 1966

Table 2. Bacterial strains used in this study.

Plasmids were from Invitrogen BV (9704 CH Groningen, The Netherlands).

2.1.4 TOPO TA Cloning Kit

All kit contents are described in the instruction manual 'TOPO TA Cloning Kit for Sequencing - Five-minute cloning of *Taq* polymerase-amplified PCR products for sequencing' Version E2 (Catalog nos. K4575-01, K4575-J10; K4580-01; K4580-40) from Invitrogen Corp. (Carlsbad, California 92008, USA).

## 2.2 General Methods

### 2.2.1 Centrifugation

Small samples were centrifuged in a bench top microfuge (Eppendorf 5417 C) using a swing-bucket rotor (Eppendorf A-8-11), (Vaudaux-Eppendorf AG, 4124 Schönenbuch, Switzerland). Larger volumes were centrifuged in a centrifuge (Sigma 204) using a swing-out rotor (Sigma 11032), (VWR International AG, 8953 Dietikon, Switzerland).

### 2.2.2 High Pressure Liquid Chromatography (HPLC)

Preservative analyses used the HPLC System 600 including a 717 & 996 photodiode array detector from WATERS (Milford, Massachusetts 01757, USA).

#### Parameters for the Determination of o-Phenylphenol (OPP)

Column:	Nucleosil 120-5 C18. 250x4 mm from Macherey-Nagel
Eluent:	Water : methanol 30 : 70 (v/v)
Flow:	1.0 ml min <sup>-1</sup>
Injection volume:	10 µl
Wavelength:	206.4 nm
Temperature:	35 °C
Method set:	OPP

Parameters for the Determination of 5-Chloro-2-Methyl-4-Isothiazoline-3-one (CMIT) and 2-Methyl-4-Isothiazoline-3-one (MIT)

Column:	Nucleosil 120-5 C18. 250x4 mm from Macherey-Nagel
Eluent:	Water : methanol 50 : 50 (v/v)
Flow:	1.3 ml min <sup>-1</sup>
Injection volume:	10 µl
Wavelength:	275 nm
Temperature:	30 °C
Method set:	Kathon

Parameters for the Determination of 2-Bromo-2-Nitropropane-1,3-Diol (BNPD)

Column:	Nucleosil 120-5 C18. 250x4 mm from Macherey-Nagel
Eluent:	Water : methanol 70 : 30 (v/v)
Flow:	1.0 ml min <sup>-1</sup>
Injection volume:	10 µl
Wavelength:	220 nm
Temperature:	30 °C
Method set:	P109

### 2.2.3 Microscopy

Microscopic analyses were carried out using an OLYMPUS BX 60 (OLYMPUS OPTICAL Schweiz AG, 8603 Schwerzenbach, Switzerland) with phase contrast, a burner type U-RFL-T and exposure control PM-20. For the execution of all fluorescence analyses a filter type NB (neutral blue) was additionally used.

#### a) Sample preparation

0.2 ml White Pigment Slurry were mixed with 10 ml nutrient broth (e.g. Tryptic Soy Broth) and incubated in a shaking incubator for 15 hours at the appropriate temperature. The suspension enriched with cells was then centrifuged for 5 min. at 1000 g. Afterwards, 0.2 ml of the

supernatant were mixed with 0.8 ml disruption buffer (DB) in a microcentrifuge tube and shaken for 3 min. by means of a minishaker. Then, 0.3 ml Nycodenz were underlaid. The sample was then centrifuged for 6 minutes at 10,000 g. The supernatant was transferred into a new microcentrifuge tube and centrifuged again for 3 min. at 10,000 g. The supernatant was separated from the pellet. Afterwards, the cells were resuspended using approx. 100 µl TBS.

b) Assessment of morphology under phase contrast

5 µl of the enriched bacteria suspension were pipetted onto a microscope slide and covered with a glass cover slide. One drop of microscope oil was applied and the assessment made at a magnification of 1000x (under phase contrast).

c) Assessment of the physiological state by means of fluorescence technology

2 µl each of SYTO 13- and Propidium Iodide were prepared in a microcentrifuge tube. 16 µl of sample (isolated bacteria) were added and stored for 2 - 3 minutes in the absence of light (aluminium foil). Afterwards, 5 µl of the bacterial suspension were pipetted onto a microscope slide and covered with a glass cover slide. One drop of microscope oil was applied and the assessment made at a magnification of 1000x under fluorescence light.

d) Reagents

- SYTO 13, live-cell nucleic acid stain, 5mM solution in DMSO (Molecular Probes, order no.: S-7575)

2 µl SYTO 13 were dissolved in 90 µl distilled water and stored in a small bottle covered with aluminium foil.

- Propidium Iodide (>95% HPLC) (SIGMA, order no.: P-4170)

10 mg Propidium Iodide were dissolved in 10 ml distilled water. 100 µl of this solution were mixed with 900 µl distilled water in a small bottle wrapped with aluminium foil and stored at -20°C.

## 2.2.4 Sterilisation

Heat stable solutions were sterilised in an autoclave (Varioklav Type 300E, H+P Labortechnik GmbH, 85764 Oberschlesienheim/München, Germany) at 15 minutes / 121°C / 1 bar. Other solutions were sterilised by passing through a 0.20 µm pore size membrane. Pipette tips, tubes and instruments were sterilised in an autoclave (Varioklav Type 300E) on a steam cycle at 15 minutes / 121°C / 1 bar.

## 2.3 Microbiological Methods

### 2.3.1 Preparation of Culture Media

#### 2.3.1.1 Routine

##### Anaerobic Agar according to Brewer (Brewer, 1940)

This medium was used for culturing *Clostridium* and other anaerobic microorganisms.

Peptone from casein	10 g
Peptone from soy flour	5 g
Yeast extract	5 g
L-cystine	0.4 g
D(+)-glucose	10 g
NaCl	5 g
Sodium thioglycolate	2 g
Sodium sulfoxylate formaldehyde	1 g
Methylene blue	0.002 g
Agar-agar	12.6 g

Made to 1 litre and autoclave for 15 minutes at 121°C (pH: 7.2 ± 0.2 at 25°C).

### Beads Working Solution

This medium was used for the calibration of the impedance and fluorescent detector of CellFacts II.

One drop of AlignFlow™ flow cytometry alignment beads 2.5µm (Molecular Probes, order no.: A-7312) was suspended in 5 ml CellLyte. The solution was then stored in the absence of light in the refrigerator at 4°C.

### Calcium Carbonate Agar

This medium was used for the detection and isolation of microorganisms as well as for the determination of the Total Viable Count in Calcium Carbonate Slurries.

Calcium carbonate	400 g
Agar-agar	15 g
TTC	10 ml

The Calcium Carbonate Agar was prepared by sterilising 400 g of dried slurry in 1 litre of water, allowing it to sediment overnight at room temperature, and then adding 15 g of agar/litre to the supernatant. After autoclaving and cooling down to 45°C, 10 ml l<sup>-1</sup> of a 1% (v/v) TTC solution were added before pouring into plates.

### Columbia Agar

This medium was used for the identification of haemolytic bacteria.

bio-Polyptone	10 g
Hydrolysed animal & vegetable proteins	10 g
bio-Myotone	3 g
Corn starch	1 g
Sodium chloride	5 g
Sheep blood	5 % (v/v)
Agar-agar	13.5 g

Made to 1 litre and dissolved by heating gently while shaking. Boiled for 1 minute. Dispensed and autoclaved for 15 minutes at 121°C (pH:  $7.3 \pm 0.2$  at 25°C).

#### (DiSC<sub>3</sub>(5)) - Working Solution

Used as a lipophilic dye (viable cell dye) for CellFacts II analysis.

1.0931 mg (DiSC<sub>3</sub>(5)) (Molecular Probes, order no.: D-306) were dissolved in 1 ml ethanol. This 2 mM stock solution was diluted in ethanol in the ratio of 1:20 and then stored in the deep freezer at -20°C. The solution was stored in the absence of light.

#### Disruption Buffer

This medium was used for the release of cells from the White Pigment surface.

Tris	6.05 g
------	--------

Na <sub>2</sub> CO <sub>3</sub>	21.2 g
---------------------------------	--------

Made to 1 litre and dissolved. Adjusted pH to 7.4 using NaOH and autoclaved for 15 minutes at 121°C. Filtered through a sterile 0.2µm filter in order to reduce the particle count.

#### Malt Extract Agar

This medium was used for the detection, isolation and determination of the total viable count of fungi and yeast in dispersants.

Malt extract	30 g
--------------	------

Peptone from soy flour	3 g
------------------------	-----

Agar-agar	15 g
-----------	------

Made to 1 litre and autoclaved for 10 minutes at 121°C (pH:  $5.6 \pm 0.2$  at 25°C).



### Nutrient Broth

This medium was used for cultivating non-fastidious microorganisms.

Peptone from meat	5 g
-------------------	-----

Meat extract	3 g
--------------	-----

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $7.0 \pm 0.2$  at 25°C).

### Nycodenz

Nycodenz was used to concentrate and isolate bacteria from White Pigment Slurries by centrifugation (density gradient cushion).

13 g Nycodenz (Sigma, order no.: D-2158) were dissolved in 10 ml sterile water under aseptic conditions in a water bath at 50°C. This solution was then stored in the refrigerator at 4°C.

### Phosphate-Buffered Saline (PBS)

This buffer was used for the dilution of slurry samples for the determination of the total viable count.

NaCl	8 g
------	-----

KCl	0.2 g
-----	-------

Na <sub>2</sub> HPO <sub>4</sub>	1.44 g
----------------------------------	--------

KH <sub>2</sub> PO <sub>4</sub>	0.24 g
---------------------------------	--------

Made to 800 ml and adjusted to pH 7.4 using HCl, made up to 1 litre and autoclaved for 15 minutes at 121°C.

### Plate Count Agar

This medium was used as an inhibitor- and indicator-free culture medium for the determination of the total viable count.

Peptone from casein	5 g
Yeast extract	2.5 g
D(+)-glucose	1 g
Agar-agar	14 g

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $7.0 \pm 0.2$  at 25°C).

### RASS-Agar (Reduced Arginine Starch Salts)

This medium was used for the culture of relatively slow growing environmental isolates.

L-Arginine	0.1 g
Starch	12.5 g
KH <sub>2</sub> PO <sub>4</sub>	1.0 g
NaCl	1.0 g
MgSO <sub>4</sub> • 7H <sub>2</sub> O	0.5 g
Agar-agar	15.0 g

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $7.0 \pm 0.2$  at 25°C).

### Sabouraud-4% (w/v)-Glucose Agar

This medium was used for the cultivation and isolation of fungi.

Peptone	10 g
D(+)-glucose	40 g
Agar-agar	15 g

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $5.6 \pm 0.2$  at 25°C).

### Sporulation Medium (Pirttijärvi, *et al.*, 1996)

This medium was used for the enrichment of spore-forming bacteria.

Peptone from meat	5 g
Meat extract	3 g
MnSO <sub>4</sub> *H <sub>2</sub> O	50 mg
CaCl <sub>2</sub> *2H <sub>2</sub> O	100 mg
MgSO <sub>4</sub> *7H <sub>2</sub> O	500 mg

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH: 7.0 ± 0.2 at 25°C).

### Syto 62 - Working Solution

Syto 62 was used as a dimeric cyanine dye (dead cell dye) for CellFacts II analysis.

The 5 mM stock solution (molecular probes, order no.: S-11344) was dissolved in deionised water in the ratio of 1:50 and then stored in the refrigerator at 4°C. The solution was stored in the absence of light.

### Tris Buffered Saline

This buffer was used in the molecular cloning and as a buffer for CellFacts II analysis.

NaCl	8g
KCl	0.2g
Tris	3g

Made to 800 ml, 0.015 g of phenol red were added and the pH adjusted to 7.4 using HCl. Made up to 1 litre (in case for CellFacts II analysis: made up to 1 litre and the pH adjusted to 5.5 using HCl). Autoclaved for 15 minutes at 121°C.

### Tryptone (Peptone C, Peptone 50, Tryptone T)

Tryptone was added to certain media as a nitrogen source (pancreatic digest of casein) for isolating and cultivating fastidious and non-fastidious bacteria.

1%, 2% and 10% (w/w) solutions are soluble in distilled water. Tryptone was added in concentrations of 1 - 2% (w/w) (nitrogen: 11.4 - 13.9%, pH:  $7.2 \pm 0.2$  at 25°C)

### Tryptic Soy Agar

This medium was used as an inhibitor- and indicator-free universal culture medium with a broad range of applications.

Peptone from casein	15 g
Peptone from soy flour	5 g
NaCl	5 g
Agar-agar	15 g

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $7.3 \pm 0.2$  at 25°C).

### Tryptic Soy Broth

This medium was used as an inhibitor- and indicator-free universal broth with a broad range of applications.

Peptone from casein	17 g
Peptone from soy flour	3 g
D(+)-glucose	2.5 g
NaCl	5 g
Na <sub>2</sub> HPO <sub>4</sub>	2.5 g

Made to 1 litre and autoclaved for 15 minutes at 121°C (pH:  $7.3 \pm 0.2$  at 25°C).

### 2.3.1.2 Culture Conditions

Cultures were grown at 25°C under anaerobic conditions (Plate Count / anaerobic jar) as well as at 30°C, 37°C, 50°C and 60°C under aerobic conditions (Plate Count).

### 2.3.2 Plate Counts

Plate Counts were used to determine the Total Viable Count (TVC) in White Pigment Slurries, dispersants and water samples.

### 2.3.3 ATP

For the measurement of the ATP content in White Pigment Slurries, the Luminometer 1253 (BioOrbit OY, 20521 Turku, Finland) was used.

The assay is based upon the quantitative measurement of a stable level of light produced as a result of the enzyme reaction catalysed by firefly luciferase. The formula for the light producing reaction is shown in Figure 9:



Figure 9. Principle of the bioluminescence assay.

Dilute trichloroacetic acid (TCA) was used in combination with the pH indicator xylenol blue to extract ATP from biological material. The firefly luciferase and luciferin contained in the ATP Monitoring Reagent were so formulated as to provide a time independent light output over the concentration range of  $10^{-11}$  -  $10^{-6}$  M. The ATP standard, which contained a known amount of ATP, was used to calibrate the assay system.

The procedure was done according to the BioOrbit instruction manual (1243-247 ATP Monitoring Reagent 5000) by using the 1243-107 ATP Assay Kit.

#### 2.3.4 RedOx

The RedOx-potential was measured in order to verify trends of critical microbial contamination in White Pigment Slurries.

A Digital pH-Meter MP 225 with a RedOx-Electrode type DM 140-SC (Mettler Toledo AG, 8606 Greifensee, Switzerland) was used for the measurements.

##### Buffer Solutions:

- 220 mV (pH: 7.0) (Mettler Toledo, order no.: 51 340 065)
- 468 mV (pH: 0.1) (Mettler Toledo, order no.: 51 340 066)

##### Procedure:

The undiluted sample was put into a sample jar. To determine the RedOx-potential, the calibrated RedOx-electrode was held direct into the slightly stirred sample. The guide value (mV) was read from the pH-meter after 1 minute. After each measurement the RedOx-electrode was cleaned with 1N HCl.

#### 2.3.5 CellFacts I - Electrical Flow Impedance

CellFacts I was used to determine the Total Viable Count (TVC) in White Pigment Slurries, dispersants and water samples.

The instrument uses electrical flow impedance to measure the number and size of all particles in a sample over the size range of 0.4 - 9.6  $\mu\text{m}$ . By focusing on the typical size range of bacteria, these cells can be counted to very low levels after a relatively short incubation, typically less than 10 hours.

The cells were suspended in an electrolyte solution and pulled by vacuum through a measuring pore of a defined diameter. An alternating voltage is placed across the length of this orifice (capillary, 30  $\mu\text{m}$ ) (Figure 10).

As, in the first approximation, intact cells can be regarded as insulators there is a measurable increase in electrical resistance when a cell traverses the orifice. The quantity of this increase depends on the volume of the cell. The voltage pulse is directly proportional to the volume. By incubating the samples small differences in the growth of the cells can be measured.

As the growth of the cells is exponential it is possible to extrapolate the number of growing organisms back to the original number of viable cells once the culture is in exponential growth.

The measurement itself takes from 40 - 60 seconds, and test results can be shown on a VDU, saved to a disk or network and printed immediately.

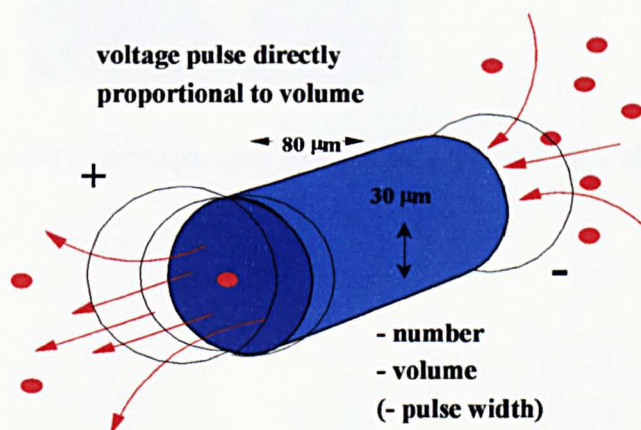


Figure 10. The operating principle of CellFacts I. It utilises electrical flow impedance to determine the concentration and size of particles and cells in a sample i.e. a particle or cell passing through an orifice, either side of which are electrodes, displaces its own volume of electrolyte so generating a voltage pulse which is directly proportional to the particle's volume irrespective of its shape. The dynamic range of the unit is such that particles are analysed over a wide range of sizes: 0.1 to  $>450 \mu\text{m}^3$  (0.4 to 10  $\mu\text{m}$  equivalent spherical diameter).

#### Procedure:

4 ml DB/TBS were added to 1 ml White Pigment Slurry in a 5 ml tube and shaken for 3 minutes on a minishaker. 300  $\mu\text{l}$  Nycondenz were prepared in a 2 ml microcentrifuge tube and 1 ml of the sample (in DB / TBS) was then carefully pipetted onto the Nycodenz. The sample had to be layered on to the Nycodenz, i.e. mixing must not occur. The samples were then centrifuged for 6

minutes at 10'000 g in a centrifuge with a swingout rotor. The supernatant was removed (Figure 11) and measured according to the working instructions for CellFacts I (CellFacts Instruments Ltd, Coventry CV4 7HS, UK) by means of the CellFacts I Analyser.

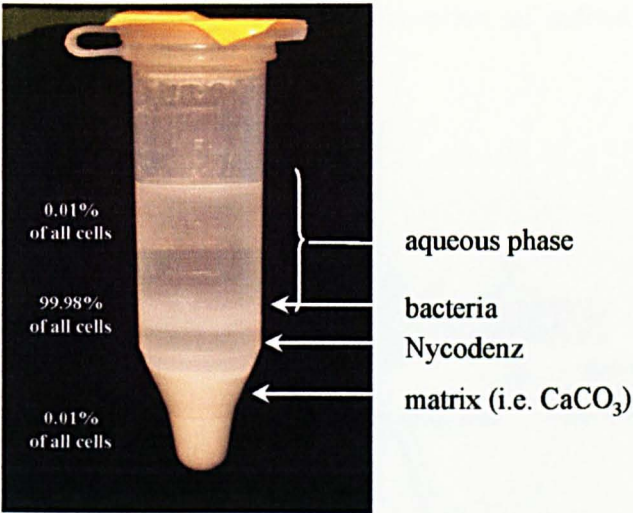


Figure 11. The Nycodenz technique used to separate bacteria from a slurry by centrifugation.



### 2.3.6 CellFacts II - Electrical Flow Impedance + Fluorescence

CellFacts II uses patented technology integrating electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics of particles i.e. cells. This is used to determine the viability, physiological state and speciation of individual particles or cells in a conductive fluid (Figure 12).

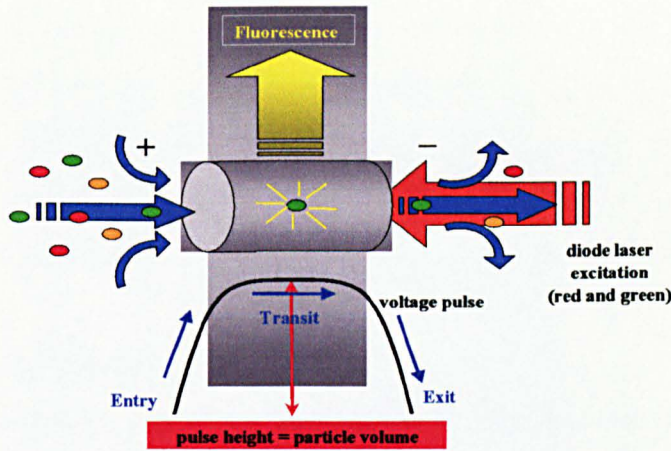


Figure 12. Schematic presentation of the CellFacts II principle. Particles (or cells) in a conductive fluid pass through a 30  $\mu\text{m}$  diameter orifice which has been laser etched in a 80  $\mu\text{m}$  sapphire disk.

Two diode lasers (red and green) are focussed on the orifice. As a particle (or cell) enters the orifice it displaces its own volume of electrolyte and this generates a voltage pulse, the magnitude of which is directly proportional to the volume of the particle. This signal is analysed in considerable detail via the software and generates information on particle (or cell) size and the numbers within a given population. It may also be used to trigger fluorescence characterisation of the particle as it transits the orifice, i.e. the integration of impedance and fluorescence data. A key feature is the orifice block detection systems which ensures the instrument operates irrespective of the size heterogeneity of the particles on the sample side of the orifice and results in an extremely effective anti-blocking system (Schwarzentruher, 2002).

#### Procedure for Coarse White Pigment Slurries:

1 ml of the slurry sample to be tested was mixed in a sterile tube with 4 ml DB and vortexed for 3 minutes. 1.5 ml each thereof was placed into two microcentrifuge tubes and centrifuged at 1000 g for 2 minutes. The upper aqueous layer (~ 1 ml) was decanted and placed into a new microcentrifuge tube that contained 30 µl of 100 µM (DiSC<sub>3</sub>(5)) and the other aliquot into a microcentrifuge tube that contained 10 µl of 500 µM Syto 62. After mixing, the samples were incubated in the dark for 8 minutes (DiSC<sub>3</sub>(5)) and 30 minutes (Syto 62) respectively. Following the appropriate incubation period, the tubes were centrifuged at 10'000 g for 3 minutes. The supernatant was then carefully decanted and discarded. 1 ml of 10 mM TBS (pH 5.5) was added to the pellet and well mixed. The analysis with the CellFacts II Analyser was as per the CellFacts II working instructions (CellFacts Instruments Ltd., Coventry CV4 7HS, UK).

#### Procedure for Fine White Pigment Slurries:

1 ml of the slurry sample to be tested was mixed with 4 ml DB in a sterile tube and vortexed for 3 minutes. 1 ml each thereof was placed into two microcentrifuge tubes and carefully layered on the top of the Nycodenz. The samples were then centrifuged at 10'000 g for 6 minutes. The upper aqueous layer (~ 1 ml) was decanted and placed into a new microcentrifuge tube that contained 30 µl of 100 µM (DiSC<sub>3</sub>(5)) and the other aliquot into a microcentrifuge tube that contained 10 µl of 500 µM Syto 62. After mixing, the sample was incubated in the dark for 8 minutes (DiSC<sub>3</sub>(5)) and 30 minutes (Syto 62). Following the appropriate incubation period, the tubes were centrifuged at 10'000 g for 3 minutes. The supernatant was then carefully decanted and discarded. 1 ml of 10 mM TBS (pH 5.5) was added to the pellet and well mixed. The analysis with the CellFacts II Analyser was according to the CellFacts II working instructions (CellFacts Instruments Ltd., Coventry CV4 7HS, UK).

### 2.3.7 Minimal Inhibitory Concentration

#### Preventive

Sterile 50 g portions of a White Pigment Slurry were inoculated with different amounts of biocide/disinfectant (dosed active/dry). The samples were then incubated for 3 days at 30°C. After storage the samples were inoculated with 1 ml of a consortium of bacteria, isolated from White Pigment Slurries, and incubated for 24 hours - 3 days at 30°C. The total viable count was determined according to the Plate Count Method. Samples that did not show any growth ( $< 100$  cfu ml<sup>-1</sup>) were further inoculated with 1 ml of the above mentioned consortium. Not more than three inoculations were performed.

#### Curative

Different amounts of biocide/disinfectant were added to 50ml samples of contaminated White Pigment Slurry. The samples were incubated at 30°C for 24 hours, followed by the determination of the Total Viable Count according to the Plate Count Method.

## 2.4 DNA Methods

### 2.4.1 Polymerase Chain Reactions

The PCR reaction mix was made to a final volume of 50 µl with sterile water and consisted of the following: primers F and R (200 ng each), 0.4 mM of each dNTP, 5 mM MgCl<sub>2</sub>, 1x PCR buffer, 2.5 µl DMSO (to reduce PCR bias), 5 µg BSA (to reduce the inhibition effects of contamination) and chromosomal DNA (1 µl of prepared supernatant). Amplifications were performed in a Hybaid thermal cycler and hot started at 80°C with 1U *Taq* DNA polymerase after an initial denaturation at 95°C for 5 minutes. 25 cycles of 94°C (1 minute), 58°C (1 minute) and 72°C (1 minute) were carried out, with a final extension at 72°C for 10 minutes.

Primer Name	Primer Sequence (5'-3')	Position in <i>E. coli</i> 16S rDNA Gene
P <sub>Forward</sub>	AGA GTT TGA TCC TGG CTC AG	8-27
P <sub>Reverse</sub>	GGT TAC CTT GTT ACG ACT T	1473-1492

Table 3. Sequence of primers used for universal PCR on 16S rDNA.

#### 2.4.2 DNA Electrophoresis and Fragment Purification

Agarose (1% (w/v) final concentration) was melted in 1x TBE (10.8 g Tris base, 5.5 g boric acid, 0.93 g EDTA). When hand warm, ethidium bromide was added (5 µl / 100 ml), and the molten gel was poured into a Bio-Rad mini cell with a gel comb. When set, this was submerged in 1x TBE containing 5 µl / 100ml ethidium bromide. DNA samples were mixed with approx. 0.2 volumes of loading buffer (50% (v/v) glycerol, 0.25% (w/v) bromophenol blue), loaded and electrophoresed at 80 V for approx. 40 minutes. The size of the DNA fragments was determined by comparison with a 100 bp or a 1 kb ladder. DNA was visualised on a UV transilluminator and photographed using UVP Grab-It-software.

##### 2.4.2.1 Agarose

The PCR product was extracted from the gel using a Qiagen Qiaquick gel extraction kit. 4.5 µl of the cleaned PCR product was mixed with 4 µl Terminator Ready Reaction Mix and 150 ng primer F in a 0.5 ml microtube. The gene was amplified in a thermal cycler with 30 cycles of 96°C (30 seconds), 50°C (15 seconds) and 60°C (4 minutes), followed by storage of the samples at 4°C. Following the labelling reaction, DNA was precipitated before automated sequencing. The labelled product was mixed with 1 µl 2 mM MgCl<sub>2</sub>, 64 µl 95% (v/v) ethanol and 26 µl sterile distilled water. The DNA was pelleted by centrifugation (13,000 g, 20 minutes) and the supernatant removed. The pellet was resuspended in 100 µl 70% (v/v) ethanol, vortexed and centrifuged (13,000 g, 20 minutes). The supernatant was removed, and final traces of ethanol evaporated off in the thermal cycler at 90°C.

#### 2.4.2.2 Bioanalyser 2100 - DNA-Chip Technology (Agilent Technologies)

The Bioanalyser was used instead of a standard gel to visualise any DNA which had been amplified via PCR.

##### Preparation of the Gel-Dye-Mix

400 µl of the DNA gel matrix were pipetted into a 1.5 ml microtube. To this, 20 µl of DNA dye concentrate were added. After vortexing the tube the gel-dye mix was transferred to the top receptacle of a spin filter (provided with the reagent kit). The spin filter was then centrifuged at 4,000 g for 10 minutes. The filter unit was then discarded and the tube labelled.

##### Chip Preparation Procedure

The chip preparation procedure was made according to the manufacturer's instructions.

##### Loading the Markers

5 µl of the markers (provided with the reagent kit) were pipetted into the well marked with the ladder symbol and 5 µl of the markers were pipetted into the 12 sample wells.

##### Loading the Ladder

1 µl of the DNA 7500 ladder was pipetted into the well marked with the ladder symbol.

##### Loading the Samples

1 µl of each sample was pipetted into one of the 12 sample wells. The chip was then placed in the vortex mixer for 1 minute at 2400 rpm. After the vortex procedure the chip was placed in the Agilent 2100 Bioanalyser and the analysis started according to the manufacturer's instructions.

### 2.4.3 Plasmid Preparation and Cloning

DNA extracts from slurry were prepared by Nycodenz recovery and sonication. The 16S rDNA genes were amplified by PCR, as described in section 2.4.1. The products were run on a 1% (w/v) agarose gel and extracted using a Qiagen Gel Extraction Kit. 4  $\mu$ l of the purified product were cloned into a TA vector and transformed into chemically competent *E. coli* (Invitrogen TOPO TA Cloning Kit), according to the manufacturer's instructions. Plating onto LB plates with 50  $\mu$ g ml<sup>-1</sup> kanamycin ensures the growth of only those cells that have received a plasmid. Recombinants were easily selected, achieved by the insertion of the lethal *ccdB* gene into the plasmid vector, which is expressed if the plasmid is not disrupted by ligation of a PCR product.

Overnight cultures of colonies picked into LB broth with 50  $\mu$ g/100 ml kanamycin were grown, and the plasmid extracted with a Qiagen Qiaprep plasmid prep kit according to the manufacturer's instructions. An aliquot of the prepared plasmid (~ 10  $\mu$ l) was then digested with *EcoRI* in the supplied buffer reaction. *EcoRI* has a restriction site on the plasmid either side of the insertion site, so the digest is a double check for the presence of an insert of the correct size. When run on a 1% (w/v) agarose gel, two bands should be seen, one of approx. 4 kb, representing the plasmid, and one of approx. 1.5 kb - the insert (Figure 13).

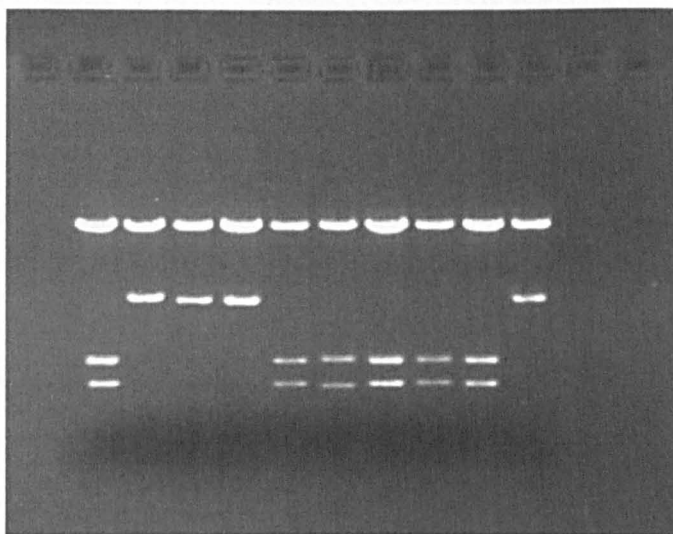


Figure 13. 10 plasmid preps digested with *EcoRI*. The top band in each lane is the plasmid (4 kb). The smaller band is the insert, and in 6 of the 10 inserts, there is a further *EcoRI* site in the insert, resulting in a double band.



Once the presence of an insert was confirmed, the DNA labelling reaction was performed as described in section 3.5, using primer M13F, which was supplied with the cloning kit. Labelled DNA was ethanol precipitated and sequenced as before.

#### 2.4.4 rDNA Internal Spacer Analysis (RISA)

In this procedure the rRNA operon is transcribed into one pre-rRNA transcript that contains the following components (5'-3') 16S, spacer, tRNA, spacer, 23S, spacer and 5S rRNA sequences with very few exceptions (Figure 14).

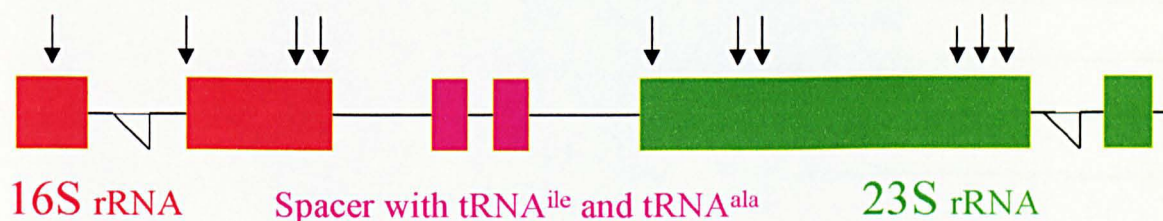


Figure 14. Diagram of beginning of rRNA operon. Arrows indicate conserved sequences, suitable for primers to be designed to (Gürtler & Stanisich, 1996).

Transcription of the operon results in the formation of a pre-rRNA transcript that is cleaved into separate rRNA and tRNA molecules. Whilst the 16S segment has been extensively studied - and 23S and 5S regions studied less so due to their size - the spacer region can also be used to yield useful information on an organism or a community. This region is extremely variable in size and sequence even within closely related taxonomic groups. There are two intergenic operons, and the variation in size is mainly due to the presence of several functional units such as tRNA genes (one or two per spacer) and recognition sites for enzymes. The functional units within the spacer do not usually sum more than 50% of its whole size and the rest of the region consists of non-essential sequences that may be less subjected to evolutionary constraints. As well as the size of the operon varying between species, the number of operons can change too, and this is also characteristic. Consequently, this heterogeneity can be used to provide a basis for identification and typing (Garcia-Martinez et al., 1999).

DNA was extracted as before and the 16S rRNA gene and/or intergenic spacer between the 16S gene and 23S gene amplified, using the PCR master mix described in 2.4.1. A range of primers was used, depending on the size of the desired amplicon, and a description of each is in Table 4.

The reaction was hot started at 80°C after an initial denaturation for 5 minutes at 95°C. Twenty-five cycles were performed of 94°C for 15 seconds, 56°C for 15 seconds and 72°C for 30 seconds, followed by a final elongation at 72°C for 1 minute (Bourneman & Triplett, 1997). The products were visualised by running on a gel prepared as before, but at a strength of 2% (w/v), and viewed by ethidium bromide staining and UV illumination or by using the Bioanalyser 2100.

Primer Name	Primer Sequence (5'-3')	Position in <i>E. coli</i> 16S rDNA Gene
28F	AGA GTT TGA TCA TGG CTC AG	8-27
1073F	ATG GCT GTC GTC AGC TCG T	1054-1072
1406F	TGY ACA CAC CGC CCG T	1390-1405
23SR	GGG TTB CCC CAT TCR G	115-130

Table 4. Universal primers for rDNA internal spacer analysis.

#### 2.4.5 Sequencing Methods

DNA precipitates were given to L. Ward (University of Warwick) for automated DNA sequencing using an ABI Prism™ 377 cycle sequencer.



## 2.4.6 Fluorescent *in situ* Hybridisation (FISH)

### Slide Preparation

Multi-well glass slides were rinsed with 70% (v/v) ethanol and then with sterile Milli-Q water, and left to dry at 60°C. 0.1125g of gelatine and 0.015g of  $\text{CrK}(\text{SO}_4)_2$  were dissolved in 150ml of water and heated to 70°C. The slides were dipped in the solution twice and allowed to dry at room temperature. The dried slides were stored in an airtight container at 4°C.

### Cell Suspension Preparation

Cell cultures/preparations in 70% (v/v) ethanol were centrifuged (13,000 g, 1 minute), the supernatant removed, and the pellet resuspended in 0.01% (v/v) NP-40 in PBS. Suspension were centrifuged again, the supernatant discarded and the pellet resuspended in 0.1% (v/v) NP-40 in water. The suspensions were centrifuged again, the supernatant discarded and the pellet resuspended in 100µl of a 1:1 mixture of storage buffer (10mM Tris-HCl pH7.4, 0.2% NP-40) and 96% (v/v) ethanol. Suspensions were stored at 4°C.

### Hybridisation

Cells suspensions were gently vortexed and 3µl of the fixed cells were applied to gelatine-coated wells and allowed to air dry. Each slide was sequentially dehydrated through a graded ethanol series of 50, 80 and 96% (v/v) for 3 minutes in each. The slides were briefly air-dried. A 9µl volume of filter sterilised hybridisation solution (0.1% (v/v) formamide, 0.9M NaCl, 0.1M Tris-HCl, 0.1% (w/v) SDS) and 1µl of labelled probe (sequences in Table 5) was spotted onto each cell preparation. Slides were placed in a pre-warmed sealed moisture chamber with a total of 900µl of hybridisation solution and incubated at 37°C for 4 hours.

Probe	Sequence (5'-3')
Non Eub 338	ACT CCT ACG GGA GGC AGC
Uni pos	TAC CGC GGC TGC TGG CA
Uni for	GGT CTG AGA GGA TGA TCA GT
Ps – for – 0289	TGC CAG CAG CCG CGG TA
Pseudo 232	TAG CTA ATC CGA CCT AGG

Table 5. Sequence of probes, all labelled with Cy5.

Following incubation the unbound probe was rinsed off with sterile Milli-Q water before incubation at 37°C in 50ml washing solution (0.9M NaCl, 0.1M Tris-HCl, 0.1% (w/v) SDS) for 20 minutes. The slides were then rinsed again in Milli-Q water and immersed in DAPI staining solution (0.9M NaCl, 0.1M Tris-HCl, 7µl/ml DAPI) for 10 minutes at room temperature. The slides were rinsed again in Milli-Q water and air-dried.

## **CHAPTER 3**

### **Microbiological Investigations - On-Site Case Studies**

### 3.1 Introduction

Detailed microbiological studies as well as analyses of White Pigment Slurries and the whole production process have never been carried out, neither by the pigment industry nor by the biocide industry. The knowledge of the microbiological flora in the products, the know-how in the area of preservation as well as of the possibilities in the area of microbiological monitoring were quite limited. In the course of this thesis, serious microbiological analysis of products and production processes on-site were for the first time carried out at the following Omya plants (in alphabetical order):

- Avenza (Italy)
- Golling (Austria)
- Gummern (Austria)
- Orgon (France)
- Molde (Norway)
- Perth (Canada)
- Verpol (USA)

The aim of the investigations was to get an insight into the microbiological diversity of the products from each plant. Identification of the sources of contamination should enable biocides to be used optimally. A further target of these investigations was to improve and standardise, respectively, the existing, partly different, monitoring systems. Furthermore, the implementation of an optimised house-keeping protocol should increase this effect.

The following results are not specific to any one plant, but reflect the trends which were generally found at the plants. The results are presented in the following sections.

### 3.2 Sources of Contamination

In the production of White Pigment Slurries there exist some critical places where bacterial contamination can occur. Often, this is due to moderate process temperatures as well as insufficient house-keeping at the respective places. Figure 15 shows a typical scheme of the final stages of the production of calcium carbonate slurries with coarse as well as fine particle size.

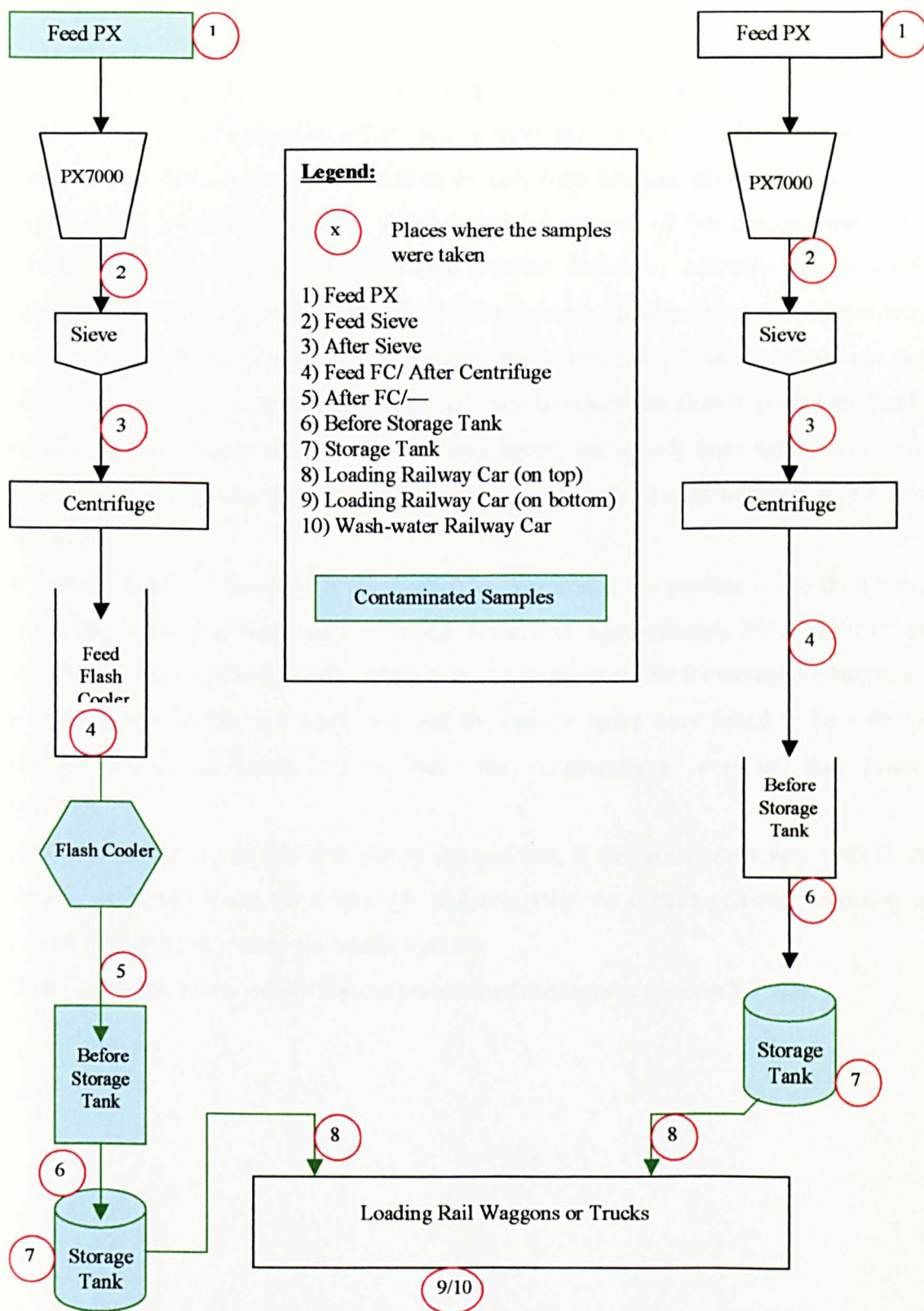


Figure 15. Schematic of the manufacturing process of coarse and fine calcium carbonate slurry.

The places marked in mint-green show where contamination can occur in the production process. Before the final grinding stage (PX) for the production of coarse calcium carbonate slurries the feed contains a high concentration of cells as these are introduced into the system through the process water (recycled water) as well as by cells from biofilms which are present in the initial stage of the production process. A bactericidal treatment of the process water as well as elimination of the biofilms before the final grinding, however, are only meaningful if thermal disinfection by high temperatures (nearly 100°C) during the final grinding is no longer achieved.

Unlike the production of coarse calcium carbonate slurries, the PX feed for the production of fine calcium carbonate slurries usually consists of material which has already passed the final grinding step for coarse calcium carbonate slurries and, hence, has already been subject to a first thermal disinfection. The results (Table 6) clearly show that the risk of contamination is quite low in this instance.

A further source of bacterial contamination in the production process is the flash cooler (FC), where the White Pigment Slurry is cooled down from approximately 80°C to 40°C. The latter temperature over a period of time offers an optimum environment for mesophilic bacteria and their growth. It is therefore not surprising that the storage tanks were found to be a further major source of contamination. Here too, the temperatures are in the lower range (> 45°C).

All the investigations carried out clearly showed that, if the temperature was > 60°C, there was usually no viable count. This strongly indicates that the microorganisms occurring in White Pigment Slurries are mostly mesophilic bacteria.

There was no evidence supporting the presence of endospores (section 5.3.1.1).



Samples Coarse CaCO <sub>3</sub> Slurry & Fine CaCO <sub>3</sub> Slurry <sup>1)</sup>	TVC (cfu ml <sup>-1</sup> ) Coarse		TVC (cfu ml <sup>-1</sup> ) Fine		Temp. °C
	3 Days <sup>2)</sup>	14 Days <sup>2)</sup>	3 Days <sup>2)</sup>	14 Days <sup>2)</sup>	Coarse/Fine
1. Feed PX	100-1000	100-1000	<100	<100	36 / 58
2. Feed sieve	<100	<100	<100	<100	82 / 73
3. After sieve	<100	<100	<100	<100	82 / 74
4. Feed flash cooler/after sieve	<100	<100	<100	<100	79 / 67
5. After flash cooler	>10 <sup>3</sup>	>10 <sup>3</sup>	—	—	41 / —
6) Before storage tank	>10 <sup>3</sup>	>10 <sup>3</sup>	<100	<100	41 / 72
7) Storage tank	>10 <sup>3</sup>	>10 <sup>3</sup>	800	1000	36 / 44
8) Filling rail waggons (with biocide)	<100	<100	<100	<100	45, 29 / 44
9) Discharge rail wag. (with biocide)	<100	<100	<100	100	39 / 37
10) Wash water (rail waggon)	>10 <sup>4</sup>	>10 <sup>4</sup>	>10 <sup>4</sup>	>10 <sup>4</sup>	23 / 29

<sup>1)</sup> The samples were taken three times and spread onto plates.

<sup>2)</sup> Incubation period of plates

Table 6. TVC of coarse and fine CaCO<sub>3</sub> slurries during production showing sources of contamination. The sensitivity of the method employed was at 100 cfu ml<sup>-1</sup>.

As mentioned before, the recycled water is the major source of contamination for the whole of the production process of White Pigment Slurries. The water isolated from the rain collecting tank, the flotation process or the return from the rail waggon cleaning plant comes into a thickener where the primary mineral particles are separated from the water by sedimentation. The more of these systems which are run in a closed circuit, the more the conditions resemble those of a microbiological fermenter. Promotion of resistance or the introduction of microorganisms which are not usually found in White Pigment Slurries are the consequences. The exact causes as well as possible solutions concerning the formation or prevention, respectively, of resistant bacteria are discussed in sections 3.5.1 as well as 5.3.1.

Several studies were carried out to identify the source(s) of contamination within the water circuit of the individual production sites. Figure 16 again shows a general scheme of treated water which eventually runs back into the production process or is used for cleaning the rail waggons, trucks or ships.

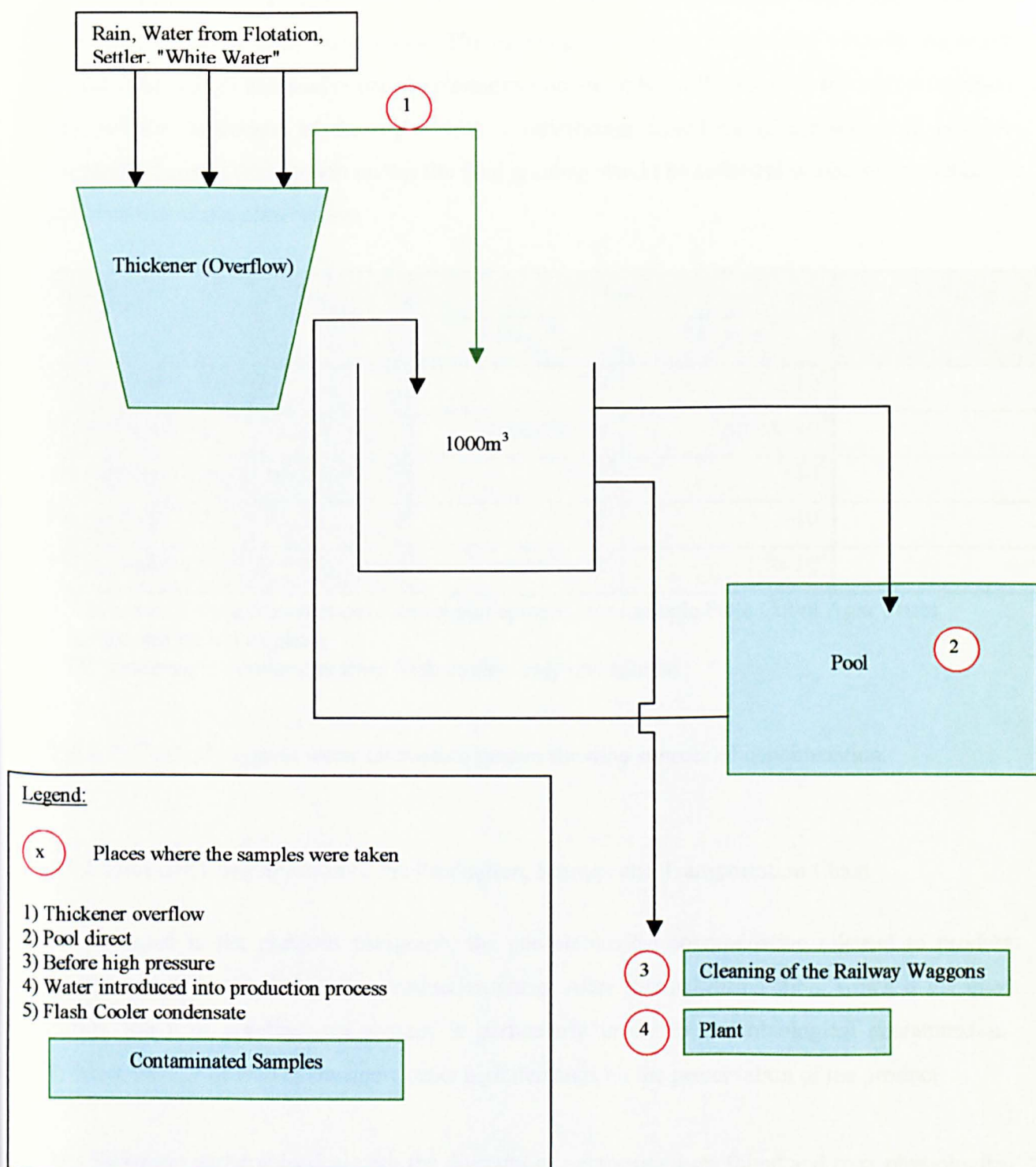


Figure 16. Schematic presentation of a water circulation within a production site.



The places marked in mint-green in figure 16 were where samples were taken for the determination of the total viable count. The results given in table 7 show that virtually the whole circuit yields a high bacterial count. As mentioned before, it has to be assessed for each production site, whether treatment of the water with a disinfectant would be advantageous or whether successful thermal disinfection during the final grinding would be sufficient to ensure the optimum performance of the preservative.

Samples H <sub>2</sub> O	TVC (cfu ml <sup>-1</sup> )		Temp. °C
	3 Days <sup>2)</sup>	14 Days <sup>2)</sup>	
1) Thickener overflow	>10 <sup>4</sup>	>10 <sup>4</sup>	28
2) Pool direct	approx. 10 <sup>5</sup>	Approx. 10 <sup>5</sup>	22
3) Cleaning of the rail waggons	>10 <sup>4</sup>	>10 <sup>4</sup>	29
4) Plant	>10 <sup>4</sup>	>10 <sup>4</sup>	27
5) FC condensate <sup>3)</sup>	approx. 10 <sup>3</sup>	1,5x 10 <sup>3</sup>	—

<sup>1)</sup> The samples were drawn once or twice and spread onto multiple Plate Count Agar plates

<sup>2)</sup> Incubation period of plates

<sup>3)</sup> FC condensate: condensate from flash cooler, only one sample

Table 7. TVC of a typical water circulation system showing sources of contamination.

### 3.3 Microbial Characterisation of the Production, Storage and Transportation Chain

As discussed in the previous paragraph, the microbiological contamination relevant to product quality occurs at the end of the production chain. After thermal disinfection, which is achieved during the final grinding, the system is particularly prone to microbiological contamination. Product storage as well as transport make high demands on the preservation of the product.

The following paragraphs deal with the diversity of microorganisms found and their physiological properties.

### 3.3.1 Total Viable Count

In all studies carried out the TVC was determined using either the appropriate agar plates or the CellFacts I as well as the new CellFacts II methods.

Since the correlation between these three measuring methods was very high, it has not been mentioned in the table which of the methods described under section 2.3 was used for the measurement.

#### 3.3.1.1 Aerobes

The presence of aerobes is shown in the example of the process study carried out at the Verpol plant (USA). Here too, it is mostly possible to draw parallels to studies carried out at other plants.

At the production site in Verpol, no growth was detected after the grinding stage (PX). In some tanks, however, bacterial growth was present. Table 8 shows that it cannot be ruled out that there is a connection between sample temperature and the potential for bacterial growth. Generally, no bacterial growth was found at temperatures above 70°C. At temperatures between 60°C and 70°C strongly reduced growth (compromised cells) was detected and at temperatures below 50°C significant growth was present, except for those storage tanks which had been treated with a preservative and where the process water had been treated with a disinfectant.

Several of the water samples were also analysed for their potential for bacterial growth. It was shown that the 11K tank as well as the warm water dilution (WWD) tank and the railcar wash water (taken from the railcars) were relatively clean. The water from 11K and WWD is mainly used before the PXs and, therefore, should not cause any problem, not even if it were slightly contaminated. The water which is used for the railcar washing is treated with a disinfectant and is clean ( $<10 \text{ cfu ml}^{-1}$ ). Contaminated water only causes a problem if it comes into contact with the product after the PX. It was also noticed that there are some closed loops in the production process. As all of these are before a PX they can be regarded as being harmless with respect to contamination. Nevertheless, closed loops have to be watched carefully as they are potential sources of aerobic and anaerobic contamination.



Samples	TVC (cfu ml <sup>-1</sup> )/day 1	TVC (cfu ml <sup>-1</sup> )/day 3	TVC (cfu ml <sup>-1</sup> )/day 4
	PCA 48h / 10d <sup>1)</sup>	PCA 3d / 10d <sup>1)</sup>	PCA 48h / 10d <sup>1)</sup>
1) 11K (disinfected)	<10 (40°C)	—	<10 (32°C)
2) Thick. Feed	>10 <sup>5</sup> (42°C)	—	>10 <sup>4</sup> (39°C)
3) T16	>10 <sup>4</sup> (42°C)	>10 <sup>3</sup> (37°C)	>10 <sup>3</sup> (35°C)
4) WWD	>10 <sup>5</sup> (35°C)	<10 (29°C)	<100 (66°C)
5) OPQ PX	<100 (81°C)	—	—
6a) OPQ PX	<100 (74°C)	—	<100 (75°C)
6b) OPQ PX	—	—	<100 (70°C)
7) T8	<100 / 400 (67°C)	>10 <sup>4</sup> (yeast) (44°C)	100 / 10 <sup>4</sup> (60°C)
8) Before T11 <sup>2)</sup>	—	<100 / 3300 (62°C)	>10 <sup>4</sup> (—)
9) T11 (preservative)	<100 / 100 (40°C)	—	<100 (45°C)
11) T0	300 / >10 <sup>4</sup> (47°C)	>10 <sup>4</sup> (44°C)	>10 <sup>4</sup> (41°C)
12) T27	10 <sup>5</sup> / >10 <sup>5</sup> (30°C)	—	>10 <sup>4</sup> (35°C)
15) H60 PX	<100 (84°C)	—	<100 (76°C)
16) 9L, H60 PX	<100 (83°C)	—	<100 (77°C)
16) 7E, H60 PX	—	—	<100 (80°C)
17) T6	<100 (78°C)	<100 (60°C)	<100 (75°C)
20) T10	—	<100 (68°C)	>10 <sup>4</sup> / >>10 <sup>4</sup> (46°C)
21) T24 (preservative)	<100 (41°C)	<100 (52°C)	<100 / 4400 (37°C)
22) OMF PX	<100 (87°C)	<100 (87°C)	—
23) OMF PX	<100 (84°C)	<100 (80°C)	<100 (84°C)
24) T2	<100 (75°C)	—	<100 (82°C)
28) RCW (disinfected)	—	<10 (41°C)	—

<sup>1)</sup> PCA: Plate Count Agar. The plates were incubated for 10 days. The evaluation was performed after 48 hours and after 10 days

<sup>2)</sup> A repeat sample was drawn two days later. The temperature of the sample was 75.5°C and the growth after 10 days was < 100 cfu ml<sup>-1</sup>.

Table 8. TVC of White Pigment Slurries and water samples from the production process at Verpol.

### 3.3.1.2 Anaerobes

Evaluation of the results of all the studies carried out at the production sites clearly showed that anaerobic conditions are very rarely found. The introduction of oxygen during and after production as well as during storage and transport is so high (due to continuous stirring or agitation) that it is unlikely that strict anaerobes will grow or indeed survive.

One problem for the White Pigment Industry as well as for other industries is corrosion. Corrosion in storage tanks and pipes can impair the production of White Pigment Slurries since rust particles in a slurry can cause considerable damage e.g. on a paper machine.

One of the causes of corrosion of metals is the formation of so-called local elements which are supported by a high electrolyte content in the surrounding solution. This electrolyte content can be formed both by chemical as well as microbial reactions. If two tangent metals (= electrically bound metals) are in contact with an electrolyte, electrons flow from the “less precious” to the “more precious” metal due to the potential difference. Thereby the “less precious” metal dissolves whereas on the “more precious” metal ions of the electrolyte (mainly  $\text{H}_3\text{O}^+$ ) are reduced. The electron transport is performed by electrolytes (catalysts).

Local elements can also form between grains of the same piece of metal/alloys or between grains and grain boundaries which usually have a somewhat varying composition. Among other things, such local elements are a cause of “pitting”.

Corrosion often occurs at “interphases”, i.e. in a storage tank where the (water-containing) product is in contact with the air (oxygen) and the metal (ring-shaped pattern of corrosion) as corrosion is mainly caused oxidatively. Corrosion can also be promoted by fine metal abrasion. Due to the large surface these small metal particles can easily oxidise (again through oxygen dissolved in water or oxygen in air) and thus lead to the formation of local elements on “clean” metal surfaces. This is due to the potential difference between different metal oxides and “clean” metals.

Some sulfate-reducing bacteria can cause an indirect anaerobic corrosion of iron. In a moist atmosphere the following reactions take place (Mortimer, 1987):

Iron oxidation:



Normally, the iron is protected from further corrosion by a film of hydrogen. When sulfate and desulfurising bacteria are present, they cause a cathodic depolarisation, and the iron is oxidised even in the absence of oxygen:

Sulfate reduction



Precipitation of iron



The sum of these three reactions:



Considerable damage to pipelines is produced in this way. *Desulfovibrio* in particular is known to be responsible for causing this kind of damage (Brock, 2000; Cypionka, 2000).

By changing the pH it is possible to influence the potential between two elements, i.e. to either prevent or promote corrosion (Nernst equation).

Further possibilities to prevent corrosion are to adapt the steel quality, to optimise the chemistry that is used or to change to other materials.

The microbiological investigations performed in Perth (Canada) clearly show that sulfate-reducing bacteria are only found in the wash water canal leading to the thickener feed tank as well as in the thickener feed tank and in the thickener (over- and underflow) itself. No ( $< 10 \text{ cfu ml}^{-1}$ ) SRBs were detected in the tanks or in the PX.

The microbes found were analysed under the light microscope (Gram stain). These microbes were found to be mainly Gram-negative rods (results not shown).

Sulfate-reducing bacteria usually grow at temperatures of between 20°C and 30°C, although there are exceptions (thermophiles). Bacteria that can grow at high temperatures are normally restricted to extreme environments. It can therefore be assumed that they do not pose a significant problem for Omya. Furthermore, they grow better in an environment with a low redox potential (- 200

mV). The redox potentials measured are relatively high, especially if one considers that 3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione, used as a preservative at the plant, decreases the redox potential. The temperatures of the samples containing SRBs are in the expected range (approx. 20°C). It is important to distinguish between bacterial growth and bacterial survival. SRBs are able to survive in less than optimal conditions (Hurst *et al.*, 2002).

Basically it can be said that most SRBs are obligate anaerobes, thus they are only found in the lower layers of deposits or at the base of biofilms. Stirred, agitated products are unlikely to be suitable for SRBs to develop.

Based on the data (Table 9) it can be assumed that the sediment of the canal leading from the railcar wash site to the thickener feed tank is a ready source of SRBs. It is also “refreshed” with nutrients on a regular basis. The microbes are then transferred from the wash water canal into the thickener feed tank where sulfate-reducing bacteria in the water phase can survive but not grow (too high redox potentials). However, in the case of tank sediments it might even be that the bacteria can grow. They would then be transferred from the thickener feed tank into the thickener where they could reproduce and grow under ideal conditions: low redox potential, optimal temperature, sufficient sulfate and other nutrients being available). In this way, the thickener is continuously re-inoculated with SRBs.

No SRBs were found in the tank, although this does not necessarily mean that there are no SRBs at all. In the case of sediments or biofilms they might occur in very small numbers or the sampling procedures were inadequate.

It cannot be ruled out that SRBs are present in sediments in the rail wagon where there is a readily available source of sulfur, for example from the breakdown products of the 3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione.

The waggons are first washed with hot water to remove the  $\text{CaCO}_3$ . Then cold, microbially contaminated, water is used. This procedure has two main effects. Firstly, the railcars are basically loaded with bacteria prior to being filled with the “clean” product. Secondly, if the wash water is repeatedly run through the same ring line, there is the potential for accumulation of different bacterial species in the wash water canal. The contaminated water therefore carries the potential for biofilm formation and the introduction of bacteria into the ring line where they reproduce. This phenomenon could be restricted by heating the wash water to 100°C for a longer period of time



and using this water for cleaning the rail wagons.

SRBs can use other substrates than sulfate as electron donor. The question is where are these nutrients, which could support the growth of SRBs provided in the system. It cannot be ruled out that decomposition products of 3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione are such a source. Furthermore, it has to be pointed out that the system is a complex one. Different bacteria often form syntrophic communities enabling each other to grow.

Samples	TSC 48h-5d	S / Dry	Cl / Dry	Redox Potential	Temp.
	cfu g <sup>-1</sup>	ppm	ppm	mV	°C
1) Wash water (canal)	< 10	187 (wet)	329 (wet)	-92	60
2) Wash water (canal)	300	—	—	-36/25	25
3) Wash water (canal)	> 3000	331	557	-55	—
4) T17	<10	553	166	+110	40
5) T16	<10	304	200	+120	40
10/28) T20	<10	78	148	40/100	70/40
11/24) Thickener feed tank	4000	180 (wet)	223/307 w	170/20	20
12/34) T35	< 10	48	109	195/82	25
13/31) T18	< 10	44	96	34/87	28
15/32) T21	< 10	149/204	158	-107/-114	15/55
16/33) T22	< 10	107	188	28/25	60/30
17) T23	< 10	76	147	-37/-73	40
18) Thickener overflow	3000	—	—	-93	—
19) Thickener overflow	> 3000	217 (wet)	251 (wet)	+82	—
20) Thickener overflow	> 3000	—	—	+130	20
21) Thickener overflow	500	178	261 (wet)	+18	—
22/35) Thickener underflow	60 / >> 3000	114/193w	118/215 w	-195/-285	20
23) PX	< 10	74	148	+120	85
25) ww canal	> 3000	—	—	-80	20
26) ww canal	3000	—	—	—	25

27) ww canal	> 3000	—	—	—	20
29) Railcar sump	140	189 (wet)	299 (wet)	+70	20

Table 9. Evaluation of the presence of sulphate reducing bacteria.

### 3.3.2 Physiological State of Individual Cells

A considerable disadvantage of the application of traditional methods such as the plate count is that they do not give any information on the physiological state of the cells. However, this information is of vital importance for testing the bactericidal effectiveness of preservatives. In fact, it occurred in the past that, after the addition of a preservative into a White Pigment Slurries, the plate count method did not show any microbiological activity but shortly afterwards, on arrival of the product at the customers, microbial contamination was detected.

This phenomenon is explained as follows: Cells that are physiologically "inactivated" (compromised / stressed cells), but not destroyed, by the active biocidal agent can recover depending on the circumstances. With biological degradation of the preservative conditions are restored which allow the microbes to become physiologically active and subsequently can be detected by the traditional methods.

A method which guarantees that an active agent produces a bactericidal action against all the microorganisms that are present in a system would not only ensure a good performance of the preservative but also optimise the costs for a plant.



### 3.3.2.1 Fluorescence Microscopy

As mentioned above in the case of ecological investigations, the customary methods for the determination of the number of cells do not show whether the detected microorganisms are physiologically active in the place where they are naturally occurring, only that they are active under the specific culture conditions employed. There is no doubt that the number of microorganisms which are actively metabolising in a mineral dispersion is considerably higher than the number found by the standard methods for the determination of the total viable count. On the other hand, it is often found that, in their natural habitat, a considerable number of the counted cells are in a state of rest and are physiologically inactive. For this reason, a series of staining methods have been developed by which it was possible to recognise viable, metabolically active, microorganisms directly under the microscope, and to distinguish them from dead or inactive cells (Figure 17 & 18) (Fry, 1990; Hall *et al.*, 1990; Lloyd & Hayes, 1995). Fluorescent dyes are preferably used for vital staining, as fluorescent cells are considerably easier to recognise and count than non-fluorescent ones.

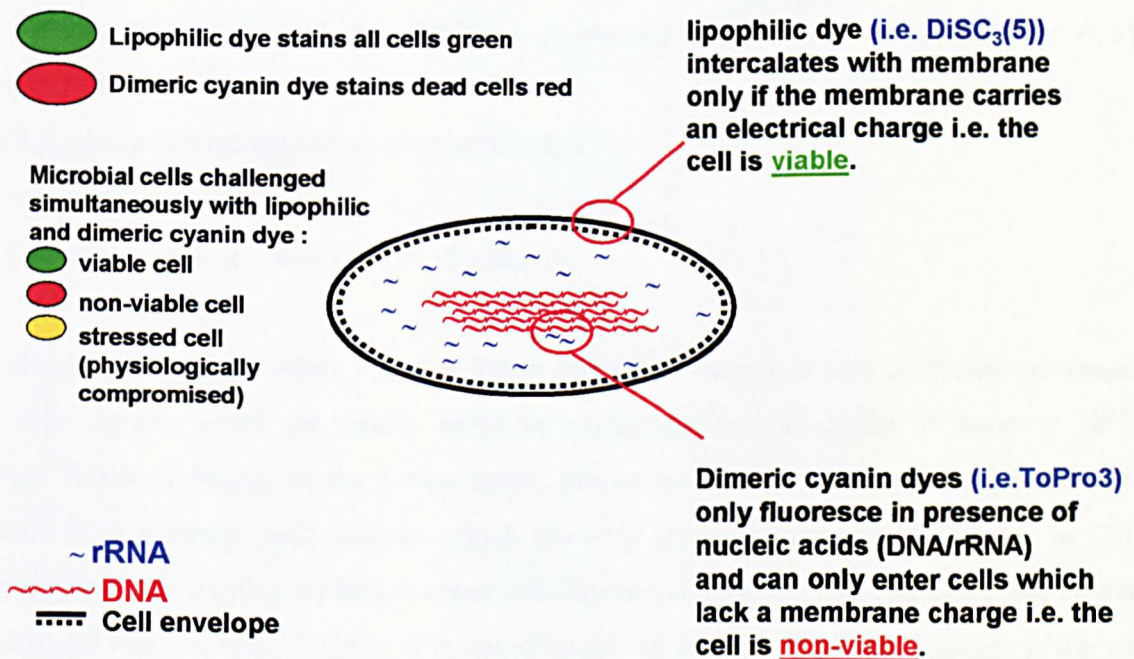


Figure 17. Staining regime used to gain information about individual cell physiology.

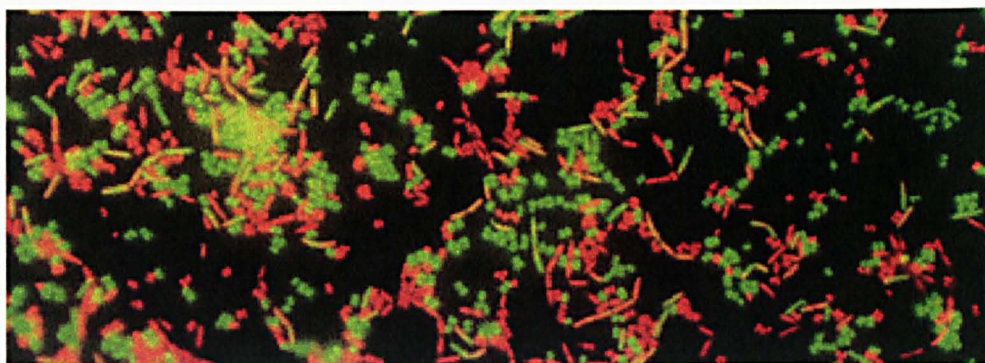


Figure 18. Viable, dead and compromised cells isolated from a  $\text{CaCO}_3$  slurry from Molde.  
(Green = viable cells, Red = dead cells, Orange = compromised cells)

In a study, the bactericidal properties of different active agents were compared by means of the fluorescence technique. At the production site in Orgon (France) it was observed that Biocide A was no longer showing a bactericidal effect and deliveries to customer were out of specification. The following substances were used in this study:

- 2-methyl-2H-isothiazole-3-one (MIT) / 5-chloro-2-methyl-2H-isothiazoline-3-one (CMIT)  
(CMIT:MIT = 3:1)
- [1,2-ethanediylbis(oxy)]-bis-methanol (EGH)
- o-phenylphenol (OPP)
- 2-bromo-2-nitro-propane-1,3-diol (Bronopol)

For this purpose, sterile White Pigment Slurry samples were mixed with controlled quantities of the active agents, which are usually added in production, and stored for 24 hours at  $30^\circ\text{C}$  to achieve thorough mixing of the active agent. The samples were then inoculated with bacteria isolated from aqueous paint samples which are very similar to the microbes found in  $\text{CaCO}_3$  slurries. The evaluation used phase contrast and fluorescence microscopy, samples being viewed at 15 min., 30 min., 60 min., 2 hours, 4 hours, 6 hours and 8 hours. Both the decrease of the viable bacteria as well as the increase in the dead bacteria were judged (Table 10). In this study the live-cell nucleic acid stain SYTO 13 (green) as well as the dead-cell nucleic acid stain propidium iodide (red) was used.



Preservative	Time (Min)	Viable Cells (Green)	Stressed Cells (Orange)	Dead Cells (Red)
MIT/CMIT	0	Yes	Yes	No
MIT/CMIT	15	Yes	Yes	Yes
MIT/CMIT	30	Yes	Yes	Yes
MIT/CMIT	60	Yes	Yes	Yes
MIT/CMIT	120	No	No	Yes
EGH	0	Yes	No	No
EGH	15	Yes	Yes	No
EGH	30	Yes	Yes	Yes
EGH	60	Yes	Yes	Yes
EGH	120	Yes	Yes	Yes
EGH	240	Yes	Yes	Yes
EGH	480	Yes	Yes	Yes
EGH <sup>a)</sup>	1800	Yes	Yes	Yes
OPP	0	Yes	Yes	No
OPP	15	Yes	Yes	Yes
OPP	30	No	No	Yes
Bronopol	0	Yes	No	No
Bronopol	15	Yes	Yes	No
Bronopol	30	Yes	Yes	Yes
Bronopol	60	Yes	Yes	Yes
Bronopol	120	Yes	Yes	Yes
Bronopol	240	Yes	No	Yes
Bronopol	360	No	No	Yes

<sup>a)</sup> To detect a possible deficiency in the action of the biocide, the samples were again measured 30 hours later.

Table 10. Bactericidal performance of preservatives on a bacterial population from Orgon. Here, where both the live-cell nucleic acid stain SYTO 13 as well as the dead-cell nucleic acid stain Propidium Iodide reacted, physiologically compromised (orange) cells could be seen. This is due to the fact that these cells have become partially permeable to Propidium Iodide which in combination with SYTO 13 yields an orange colouration.

Table 10 shows that [1,2-ethanediylbis(oxy)]-bis-methanol has a deficiency in its action against one of the bacterial species present at the Orgon plant and used in this study. This is also clearly seen in Figure 19 where one type of bacterial cell still shows viability, even after 30 hours of contact with the active biocide. This is not necessarily recognised by the traditional methods due to their culture selectivity. Furthermore, it was possible to show that the fastest bactericidal action is achieved with OPP, whereas with Bronopol the bactericidal effect only started after 6 hours and this was not expected.

The reason for the ability of [1,2-ethanediylbis(oxy)]-bis-methanol to inhibit only one of the species present in the production process at the Orgon plant is discussed in section 5.3.1.2.

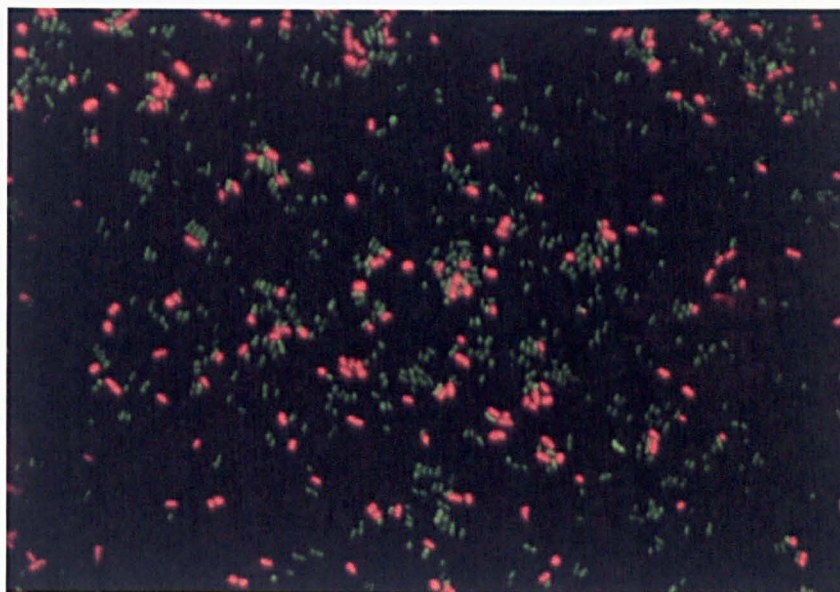


Figure 19. Deficiency of [1,2-ethanediylbis(oxy)]-bis-methanol activity on two species isolated from Orgon.

Green = viable cells of species unaffected by biocide  
Red = dead cells - consequence of biocide action

### 3.3.3 Diversity of Organisms

The decision to investigate the microbial diversity existing in White Pigment Slurries was made by consideration of a number of factors:

- It has been demonstrated that culture-based techniques only isolate ~ 0.1 % of the total microbial population in soils (Borneman & Triplett, 1997). Since only one or two colony types are generally cultured upon spread plating of White Pigment Slurries, it is conceivable that a similar situation may exist in this environment.
- Microscopic analysis of bacteria concentrated from White Pigment Slurry typically reveals a diverse range of cell morphologies.
- Preliminary 16s rRNA gene sequence analysis of slurry isolates revealed diversity greater than previously thought e.g. *Methylobacterium* sp., various *Actinomycetes*
- By using molecular techniques (mainly PCR and cloning) it should be possible to apply techniques that permit the *in situ* detection and enumeration of organisms of commercial interest (i.e. bacteria that have the potential to compromise slurry quality), while establishing a database of the microbial consortia typically (or otherwise) present in the slurry environment.



A number of molecular techniques were selected to aid in the identification of the slurry population which are summarised in Figure 20:

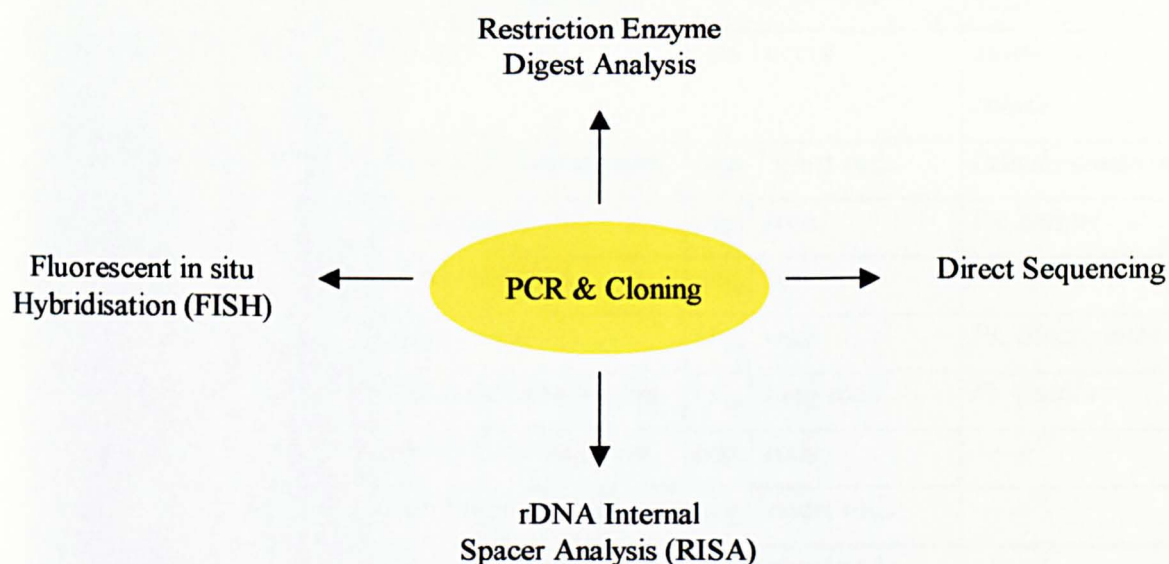


Figure 20. Molecular techniques to aid in the microbial characterisation of a slurry population.

### 3.3.3.1 Plate Count

As mentioned before, the determination of species diversity in a White Pigment Slurry by means of different culture media is limited due to selectivity. Characterisation of species diversity by means of culture media alone would therefore not be meaningful and rather time-consuming. Nevertheless, it is possible to obtain an impression of the microbiological diversity in White Pigment Slurries as well as of dispersants by using the traditional culture and Gram-staining procedures. However, microorganisms in dispersants cannot survive in slurries if polyacrylic acid is introduced during dispersion. The alkaline pH (8 - 10) as well as the difference in the osmotic pressure (osmotic shock) do not provide a favourable environment.

Table 11 shows the different bacteria isolated from pigment slurry, water and dispersant samples.

Samples	Appearance of the Colonies (PCA)	Microscopic Data (Gram stain)	Identification
H60 (before PX) VII	White	pos. <sup>1</sup> cocci	<i>Staphylococcus cohnii</i>
H60 (before PX) XVIII	Yellowish, transparent	neg. <sup>1</sup> short rods	<i>Pseudomonas alcaligenes</i>
H60 (FC) XIV	Transparent	neg. <sup>1</sup> rods	<i>Ps. stutzeri</i>
H60 (FC) XIII	Yellowish, slimy	neg. <sup>1</sup> rods	____ <sup>3)</sup>
H60 (st. tank) XXXIII	Transparent	neg. <sup>1</sup> rods	<i>Ps. alcaligenes</i>
H60 (st. tank) XXXIV	Yellowish, transparent	neg. <sup>1</sup> long rods	<i>Ps. putida</i>
H60 (st. tank) XXX	yellowish, transparent	neg. <sup>1</sup> rods	____ <sup>3)</sup>
H60 (st. tank) XXXII	rose-like, yellowish	neg. <sup>1</sup> short rods	____ <sup>3)</sup>
SC HG XXVI	white, turns red	neg. <sup>1</sup> short rods	____ <sup>3)</sup>
SC HG (st. tank) XIX	yellowish, transparent	neg. <sup>1</sup> short rods	<i>Ps. alcaligenes</i>
SC HG (st. tank) XXXVI	transparent	neg. <sup>1</sup> rods	____ <sup>3)</sup>
SC HG (st. tank) XXV	transparent	neg. <sup>1</sup> irregular rods	____ <sup>3)</sup>
FC condensate VIII	slightly reddish	pos. <sup>1</sup> (lab.) cocci	Evt. <sup>2)</sup> <i>Kocuria varians/rosea</i>
FC condensate IX	yellowish, transparent	neg. <sup>1</sup> short rods	<i>Ps. alcaligenes</i>
FC condensate X	white, fine	pos. <sup>1</sup> cocci	<i>Micrococcus spp.</i>
Wash water RC I	white, round	pos. <sup>1</sup> cocci	____ <sup>3)</sup>
Wash water RC III	yellowish, transparent	neg. <sup>1</sup> rods	<i>Ps. putida</i>
Wash water RC XII	small, transparent	neg. <sup>1</sup> short (coccoid) rods	____ <sup>3)</sup>
Wash water RC XXXVI	slightly pink	pos. <sup>1</sup> rods of cocci	____ <sup>3)</sup>
Water pool VI	red	neg. <sup>1</sup> long rods	____ <sup>3)</sup>
Water pool XVI	fine, transparent	neg. <sup>1</sup> coccoid rods	<i>Moraxella spp.</i>
Water before high press.	transparent	neg. <sup>1</sup> rods	<i>Ps. putida</i>

BC70/70 XX	salmon-coloured	pos. <sup>1</sup> rods of cocci	<i>Micrococcus spp.</i>
BC70/70 XXI	yellowish	pos. cocci	<i>Staph. sciuri</i>
Polystabil XXII	white	pos. large „patches“	probably yeast

<sup>1)</sup> neg./pos.: Gram-negative / Gram-positive

<sup>2)</sup> Identification was made by the „Api-Test-System“ which is not appropriate in each case.

<sup>3)</sup> Identification was not made / not possible.

Table 11. Microorganisms isolated from pigment slurry, water and dispersant samples using PCA.

As was expected, traditional culturing methods showed that the White Pigment Slurries mainly contained Gram-negative rods, mostly pseudomonads. Apart from Gram-negative microbes Gram-positive cells, mainly cocci, were also found in the water samples. The bacteria found in the dispersants were also identified as Gram-positive cocci (Table 11). This confirms the above-mentioned result that a slurry is usually a poor medium for microbes introduced via dispersants.

### 3.3.3.2 Microscopy

Initial microbiological analysis of a range of White Pigment Slurries, employing traditional plating techniques, typically revealed the presence of only one or two bacterial colony types. Identification of purified colonies using the API biochemical test system revealed that most of these isolates were pseudomonad species, although interestingly, few isolates could be cultured on pseudomonad selective agar. However, direct removal and concentration of bacteria from slurry by differential centrifugation, followed by light microscopy, revealed much greater morphological variation than would be expected (Figure 21). This suggested that plating on nutritionally rich media (PCA, NA) was selectively enriching only one or two species, and was therefore of limited use in gaining a complete understanding of the microbial diversity of the slurry.



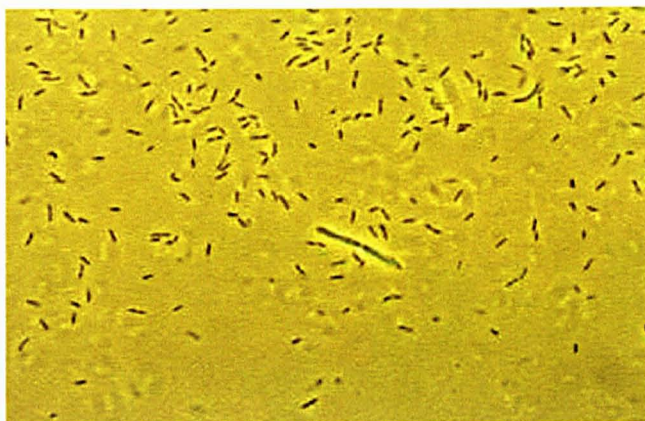


Figure 21. Phase contrast micrograph (1000x) of bacteria isolated from a calcium carbonate slurry by differential centrifugation. The diverse morphology of bacteria present indicates that the microbial population of the slurry consists of a wide range of species.

Better results were obtained when slurry samples were cultured on reduced arginine starch salts (RASS) medium, which was developed for the culture of relatively slow growing environmental isolates. Plating on this medium generally led to an increase in the diversity of colony types, including for the first time Gram-positive and filamentous organisms (Figure 22) in White Pigment Slurries from Molde and Orgon.

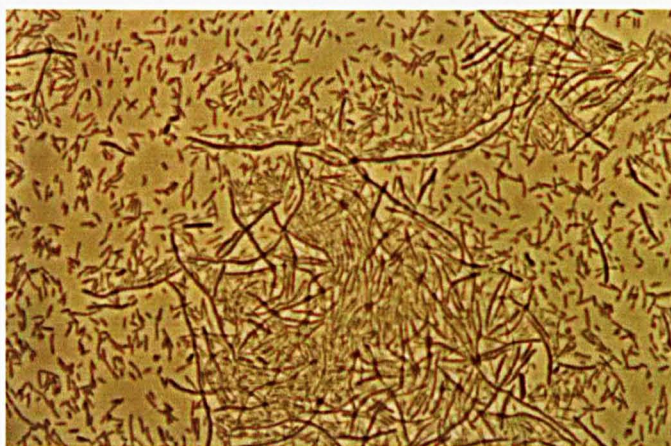


Figure 22. Light micrograph (1000x) of filamentous organism isolated from a calcium carbonate slurry (Molde). The combination of filamentous growth and spore-like structures suggest that this organism is a member of the *Actinomycetes*.

By using electron microscopy, the results obtained by light microscopy were only partially confirmed. Due to the prevailing presence of Gram-negative pseudomonads in White Pigment Slurries it was difficult to detect other morphologies under the electron microscope (Figure 23).



Figure 23. Electron micrographs showing pseudomonad types isolated in White Pigment Slurries.

### 3.3.3.3 Molecular Biology

#### 3.3.3.3.1 Phylogenetic Identification of White Pigment Slurry Microbes

Characterisation of the microbial diversity of environmental samples by analysing rRNA sequencing data is a well established technique and has a number of advantages over traditional plating techniques. The main advantage of the technique, when utilised fully, is the removal of all cultivation/isolation steps while permitting the very sensitive detection and identification of individual species in a mixed population of organisms. To date, the application of the technique to White Pigment Slurries has been restricted to PCR amplification and sequencing of 16S rRNA from slurry isolates - largely in response to the limited identification offered by the API system. Ultimately, however, it should be possible to extract "total population" DNA direct from the slurry, which can be amplified, cloned, and sequenced to obtain much more information on the diversity of organisms than that obtained by traditional methods. Information on isolates identified by comparing sequencing data with that on the NCBI database is contained in Table 12.

Bacterial species	Source	Pigment	Base Pair Alignment (% Similarity)
<i>Acidovorax delafieldii</i>	Molde	CaCO <sub>3</sub>	96
<i>Aeromonas caviae</i>	Avenza	CaCO <sub>3</sub>	98
<i>Aeromonas hydrophilia</i>	Orgon	CaCO <sub>3</sub>	97
<i>Aeromonas salmonicida masoucida</i>	Verpol	CaCO <sub>3</sub>	96
<i>Agromyces ramosus</i>	Orgon	CaCO <sub>3</sub>	98
<i>Alcaligenes xylosoxidans</i>	Perth	CaCO <sub>3</sub>	98
<i>Bdellovibrio bacteriovorus</i>	Molde	CaCO <sub>3</sub>	95
<i>Flavobacterium indologenes</i>	Perth	CaCO <sub>3</sub>	99
<i>Hyphomicrobium vulgare</i>	Orgon	CaCO <sub>3</sub>	98
<i>Methylobacterium extorquens</i>	Orgon	CaCO <sub>3</sub>	98
<i>Methylobacterium mesophilicum</i>	Avenza	CaCO <sub>3</sub>	95
<i>Moraxella spp.</i>	Gummern	CaCO <sub>3</sub>	95
<i>Micrococcus luteus</i>	Gummern	CaCO <sub>3</sub>	96
<i>Micrococcus roseus</i>	Molde	CaCO <sub>3</sub>	97
<i>Micrococcus varians</i>	Verpol	CaCO <sub>3</sub>	95
<i>Pseudomonas aeruginosa</i>	Molde	CaCO <sub>3</sub>	99
<i>Pseudomonas alcaligenes</i>	Gummern	CaCO <sub>3</sub>	98
<i>Pseudomonas cepacia</i>	Verpol	CaCO <sub>3</sub>	97
<i>Pseudomonas diminuta</i>	Perth	CaCO <sub>3</sub>	95
<i>Pseudomonas fluorescens</i>	Perth	CaCO <sub>3</sub>	96
<i>Pseudomonas luteola</i>	Avenza	CaCO <sub>3</sub>	96
<i>Pseudomonas maltophilia</i>	Verpol	CaCO <sub>3</sub>	98
<i>Pseudomonas mendocina</i>	Avenza	CaCO <sub>3</sub>	95
<i>Pseudomonas paucimobilis</i>	Molde	CaCO <sub>3</sub>	95
<i>Pseudomonas pickettii</i>	Orgon	CaCO <sub>3</sub>	95
<i>Pseudomonas pseudoalcaligenes</i>	Verpol	CaCO <sub>3</sub>	99
<i>Pseudomonas putida</i>	Gummern	CaCO <sub>3</sub>	98
<i>Pseudomonas stutzeri</i>	Gummern	CaCO <sub>3</sub>	99
<i>Pseudomonas testosteroni</i>	Molde	CaCO <sub>3</sub>	95
<i>Pseudomonas vesicularis</i>	Molde	CaCO <sub>3</sub>	95
<i>Sphingobacterium spiritivorum</i>	Orgon	CaCO <sub>3</sub>	95
<i>Staphylococcus capitis</i>	Avenza	CaCO <sub>3</sub>	95
<i>Staphylococcus cohnii cohnii</i>	Gummern	CaCO <sub>3</sub>	97
<i>Staphylococcus lentus</i>	Perth	CaCO <sub>3</sub>	96
<i>Staphylococcus sciuri</i>	Gummern	CaCO <sub>3</sub>	98
<i>Staphylococcus xylosus</i>	Perth	CaCO <sub>3</sub>	96

Table 12. Isolates identified by comparing sequencing data.

It is interesting to note that a number of the pseudomonad species isolated and identified from 16S sequence data only show 95% homology to those contained in the database (95% similarity is taken as "safe" for genus level identification, while 98% and above is accepted as "safe" to species level). This adds evidence to the idea that many of the organisms found in White Pigment Slurries may be fairly unique or unusual organisms, being some what removed from type strains and clinical isolates. The 16S data also reveals, perhaps not surprisingly, that most (if not all) of the isolates typed to date are common soil and water organisms. However, the above list is by no means complete.

#### 3.3.3.3.2 Detection of Specific Groups of Bacteria by PCR

The production plant in Perth, Canada developed a problem with bacteria suspected to be sulphate-reducers (SRBs). They caused the slurry to develop a grey coloration and strong corrosion in the centrifuges (as described in section 3.3.1.2) but could not be easily cultured due to their fastidious requirements. Consequently, a PCR based method was applied to specifically detect SRBs, i.e. slurries suspected of containing bacteria (which showed no growth on media plates) were extracted with Nycodenz, then amplified with universal 16S primers to confirm whether any bacteria were indeed present. Then, nested primers designed to amplify specific sub-groups of SRB 16S rDNA were applied in a further round of amplification using the primary PCR product as a template. The presence of specific groups of SRBs was then confirmed simply by the presence or absence of a PCR product.

Universal PCR amplification of the 16S rDNA recovered by the Nycodenz and lysis method from various slurry products and slurry intermediate products was performed, as described in section 2.4. Then, a second round of amplification of positive samples was set up, using specific primers for each group of SRB (shown in Table 13) and 2 µl of the universal PCR product as a template. Aliquots were then run on an agarose gel, as before, and the presence of a PCR product of the expected size indicated the presence of SRB genes in the universal product, and therefore also in the sample.



Primer	Target Site	Sequence (5'-3')	Specificity	Expected Product Size
DFM140	140-158	TAG MCY GGG ATA ACR SYK G	Group 1	700
DFM842	842-823	ATA CCC SCW WCW CCT AGC AC		
DBB121	121-142	CGC GTA GAT AAC CTG TCY TCA TG	Group 2	1120
DBB1237	1237-1215	GTA GKA CGT GTG TAG CCC TGG TC		
DBM169	169-183	CTA ATR CCG GAT RAA GTC AG	Group 3	840
DBM1006	1006-986	ATT CTC ARG ATG TCA AGT CTG		
DSB127	127-148	GAT AAT CTG CCT TCA AGC CTG G	Group 4	1150
DSB1273	1273-1252	CYY YYY GCR RAG TCG STG CCC T		
DCC305	305-327	GAT CAG CCA CAC TGG RAC TGA CA	Group 5	860
DCC1165	1165-1144	GGG GCA GTA TCT TYA GAG TYC		
DSV230	230-248	GRG YCY GCG TYT CAT TAG C	Group 6	610
DSV838	838-818	SYC CGR CAY CTA GYR TYC ATC		

Table 13. 16S rDNA-targeted PCR primer sequences specific for SRB subgroups (Daly, 2000).

Five of seven slurry samples were positive using the universal PCR primers. None of these samples showed any growth on agar plates. Two of these positive samples (one final product, and one from a PX feed tank) were then studied with each set of nested primers. Two sets of primers produced a product, those specific for *Desulfobulbus*, *Desulfococcus*, *Desulfonema* and *Desulfosarcina* (Figure 24).

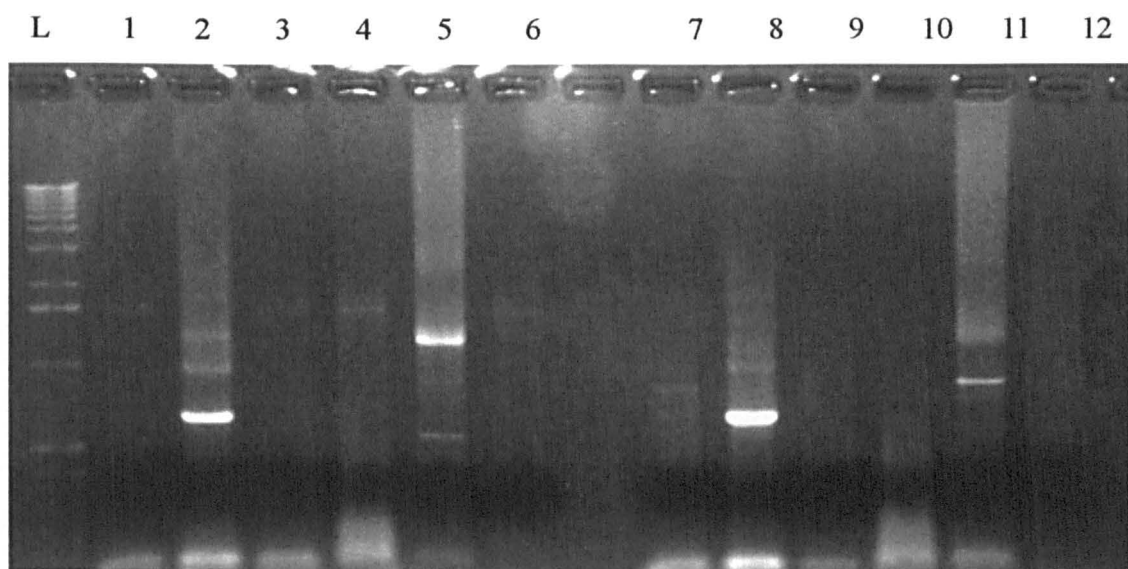


Figure 24. Two slurry samples were amplified with set of SRB specific primers in the order 1: DFM, 2: DBB, 3: DBM, 4: DSB, 5: DCC, 6: DSV. Slurry 1: lanes 1-6, Slurry 2: lanes 7-12.

This method therefore permitted the detection of low quantities of specific DNA from calcium carbonate slurries i.e. the nested approach improved the detection of low concentrations of DNA that could not be found without the two-stage PCR process. However, PCR detects DNA only, and gives no indication of the viability of cells. The bacteria may not have been culturable due to their fastidious requirements, or it may be because they are simply not viable due to the addition of disinfectants and/or preservatives. Furthermore, thermal disinfection at the fine grinding step of the production process is likely to kill a high proportion of contaminating bacteria, and it is unknown whether DNA detected by PCR comes from viable or non-viable cells. Given that the SRBs were causing the above mentioned problems in the slurry samples examined, it is likely that the cells were viable and biocide dosage should have been adjusted accordingly.

### 3.3.3.3.3 Cloning

The ability to quantify the number and species of microorganisms within a community is fundamental to the understanding of the structure and function of an ecosystem. Using simple morphology and physiological traits can often be ambiguous, and in addition, there is the problem of organisms resisting cultivation, which is essential to their full characterisation. Recombinant DNA techniques have provided a means whereby many of the obstacles associated with cultivation and description can be overcome and subsequently have allowed many new insights into the complexity of natural microbial communities. The 16S rDNA approach can be extended to include a cloning step, thus allowing the direct investigation of the community structure, diversity and phylogeny of microorganisms in almost any environment.

Essentially, the technique involves the extraction of 'total population' DNA, followed by a PCR reaction employing universal 16S DNA primers. This results in a mixed PCR product containing amplified fragments of 16S DNA from the full spectrum of slurry organisms. The next step of the procedure involves cloning of the PCR products into plasmid vectors that are themselves amplified inside an *E. coli* host (the objective here is to separate the mixed PCR products). Successful cloning is followed by selection of 20 - 50 colonies that will hopefully contain a representative range of PCR fragments from the White Pigment Slurry microbes. Plasmid DNA can then easily be purified using standard plasmid preparation kits before sequencing is performed, resulting in a library cataloguing the full range of microorganisms present in the slurry.

Several CaCO<sub>3</sub> slurries were cloned, including samples from Gummern (Austria), Molde (Norway) and Orgon (France). Species such as *Acidovorax defluvii*, *Bdellovibrio bacteriovorus*, *Methylobacterium extorquens*, *Pseudomonas stutzeri* and *Pseudomonas pseudoalcaligenes* were identified. A list of all bacteria identified is given in Table 12. Several clones were 'identified' as uncultured eubacteria. These are unculturable due to their unknown requirements but may nevertheless be important members of the community. So, although little may be known about these bacteria, they should not be forgotten, and should be included in libraries of contaminants. Whilst techniques such as PCR and cloning have undoubtedly led to new and valuable knowledge,

as with all methods, there are important limitations that must be recognised and considered. Selectivity in PCR amplification of rDNA genes is a source of bias that can affect the results of molecular biological methods of diversity analysis. Small differences in the sequence of universally conserved regions may result in selective amplification of some sequences (von Wintzingerode *et al.*, 1997). Furthermore, it cannot be assumed that the frequency of different sequence types in PCR-derived cloning represents the relative abundance of those organisms in the microbial community. The copy number of rDNA genes present within the genomes of different organisms can range from 1 to 14, so even assuming unbiased amplification the abundance of particular sequences within a clone library can be skewed. There is also concern that species that are more abundant are preferentially amplified, and low-abundance sequences are discriminated against, so if the DNA sample contains similar templates, PCR bias is greater (as product concentration is high enough to inhibit reannealing) (Suzuki & Giovannoni, 1996). As slurry biodiversity is currently unknown, but suspected to be fairly conserved, PCR bias could be strong in these samples. However, since bias is dependent on the number of cycles in the PCR reaction, keeping the cycle number low can reduce it. Cell morphology can also affect sequence retrieval, as Gram negatives are easier to lyse than Gram positives, spores are virtually impossible to lyse, and even small cells (0.3 - 1.2  $\mu\text{m}$ ) (possible in slurry due to state of starvation) are more resistant to lysis than large ones (Theron & Cloete, 2000). It has also been observed that cloned PCR libraries generated using different primers and vectors can result in differences in composition (Head, Saunders & Pickup, 1998). The implication of these findings is that we can never confidently extrapolate from sequence composition in a clone library to a quantitative population composition in an environmental sample. However, by combining the data retrieved from cloning experiments with further techniques such as *in situ* hybridisation, these problems can be overcome. Using these techniques will allow not only the determination of cell morphology of an uncultured microorganism and its abundance, but also the analysis of spatial distributions *in situ*.



#### 3.3.3.3.4 rDNA Internal Spacer Analysis (RISA) of Cultured Organisms

The main pitfall with the use of 16S rDNA for studying biodiversity is that in terms of size, the genes for the 16S molecule are relatively constant (mean length 1500bp with total variation of about 200bp), so different genes cannot be separated by size. For sequencing, the total amplicon has to be cloned and the individual clones sequenced separately. This is a methodological limitation for large-scale screening where many samples need to be compared, and in any case requires the tedious and expensive sequencing of many clones often revealing identical (or nearly so) sequences. This problem can be simplified by including the RISA region in the PCR amplification. The digested plasmid preparation in section 2.4.3 (Figure 13) is from an original PCR that only amplified the 16S gene, and all the inserts have one of two types of profile, thus offering little in terms of screening before sequencing. However, the digested plasmid prep (Figure 25) contains an insert with both the 16S gene and the RISA region and displays much more heterogeneity in profile, allowing a degree of screening when selecting which plasmids to sequence. Sequence information has also become available on the RISA region, so this can now too be used for identification.

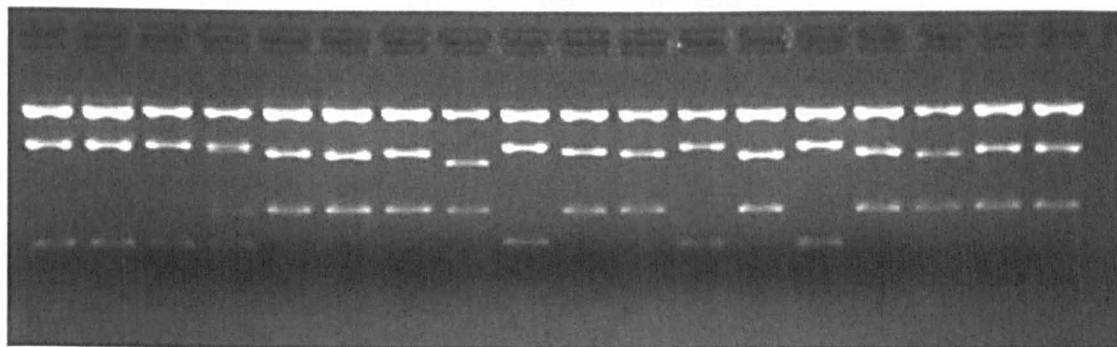


Figure 25. Plasmids from cloned isolates from slurry ex Vermont, USA, digested with *EcoRI*. By including the RISA region more double-banded inserts were present, but a difference in the sizes of these fragments is apparent, indicating the presence of a number of different species.

As well as providing a further screening step in cloning experiments, the RISA method can also be applied to community fingerprinting. A variety of White Pigment Slurries of different grades and from different plants were Nycodenz extracted, washed and lysed by sonication before PCR amplification of the RISA region either by running a gel or by the Bioanalyser 2100.

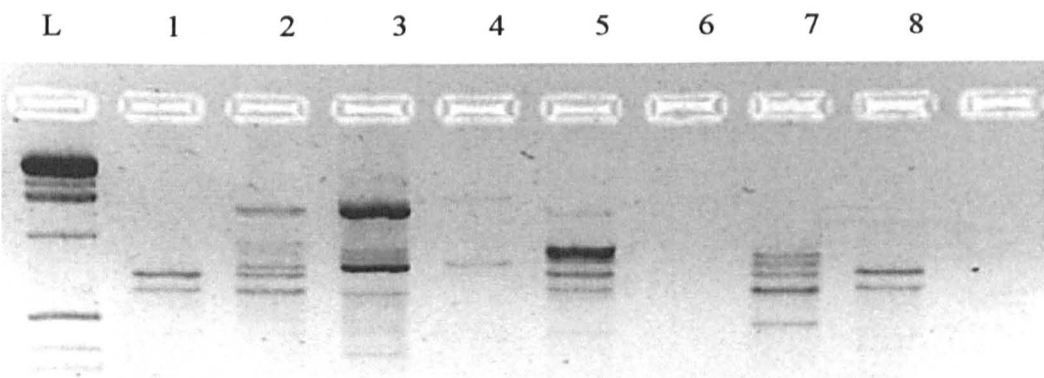


Figure 26. Amplification of the RISA region of DNA from slurries from 1: Molde, 2: Gummern (batch 1), 3: Gummern (batch 2), 4: Gummern (batch 3), 5: Avenza, 6: Orgon, 7: Vermont and 8: Perth.

Profiles in Figure 26 show a number of interesting characteristics. Firstly, it appears that there are two operons common to 6 of the 8 slurries, which may come from a single organism with two operons or two different organisms. Either way, this evidence supports sequencing and API data, which frequently finds organisms common to a number of different slurries (often pseudomonad species). The three slurries from the same plant show greater similarity than those from different sites (especially batches 1 and 2), but there is still considerable deviation, indicating that the microbial consortia inhabiting slurries changes with time. This could be important when deciding biocide dosage, which should vary according to the concentration of and type of contamination. The slurry from Orgon shows no banding pattern. However, when plated out, bacteria were present in relatively high numbers (approximately  $10^5 \text{ ml}^{-1}$ ). This plant has had a number of problems with biocide resistance and contamination with highly pigmented bacteria (found to be *Methylobacterium extorquens*), which resulted in a pink tinge to the product. It may be that the lysis technique is not sufficiently rigorous, explaining why no bands were present following

amplification. There is therefore considerable work still to be done on this technique, which provides a good example of how PCR bias can strongly influence results. In addition, it should be remembered that for community fingerprinting, the diversity that can be described is limited by the length variation in the spacer region.

A characteristic banding pattern for a number of organisms was found upon PCR amplification of the 16S-23S RISA region (Figure 26 & 27). This raises the possibility that spacer variations between strains, species and genera may be used for typing purposes.

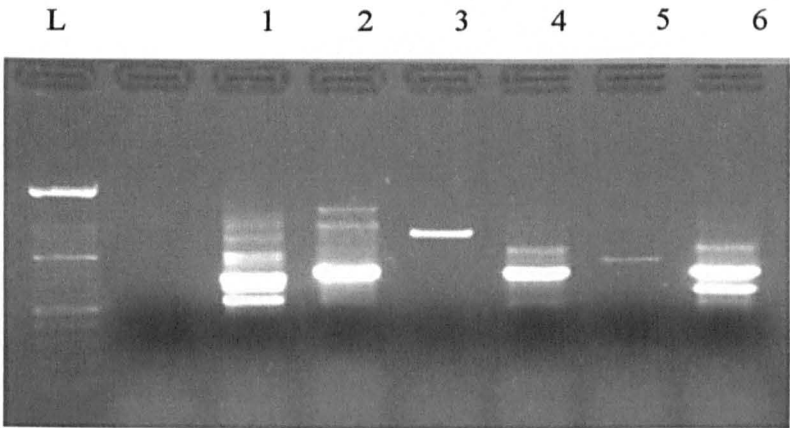


Figure 27. RISA banding patterns for 1: *E. coli*, 2: *Ps. stutzeri*, 3: a ‘smooth’ isolate, 4: a ‘wrinkly’ isolate, 5: a ‘yellow’ isolate and 6: a ‘white, resistant’ isolate.

This method offers the potential to reduce the amount of expensive and time consuming sequencing required. Instead, bacterial typing based on spacer length variation can be used. This is most reliable in species with multiple rRNA operons (3/4+). The pattern of products obtained can then be matched to a database of band patterns from common slurry strains. A basic comparison of known organisms and unknown isolates by their RISA regions is shown (Figure 28).

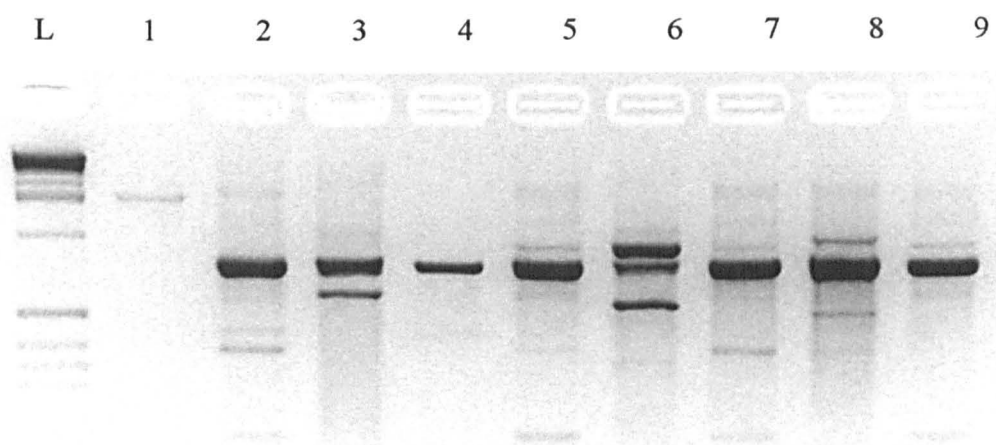


Figure 28. RISA of bacterial isolates. 1: Negative control, 2-4: 3 isolates from Gummern, 5-6: 2 isolates from Vermont, 7: *Comomonas* sp., 8: *Pseudomonas pseudoalcaligenes* and 9: *Pseudomonas stutzeri*.

From Figure 28, isolate 1 from Gummern (lane 2) shows similarity to *Comomonas* sp. (lane 7) and isolate 1 from Vermont (lane 5) is similar to *Ps. stutzeri* (lane 9).

RISA also offers the opportunity to compare a community extraction with isolates on plates. Nycodenz recoveries from two calcium carbonate slurries, Gummern and Vermont, were prepared and lysed by sonication as before. Isolates from these slurries on agar plates were also picked and lysed. All DNA extractions then underwent PCR of the RISA region, as shown in Figure 29.

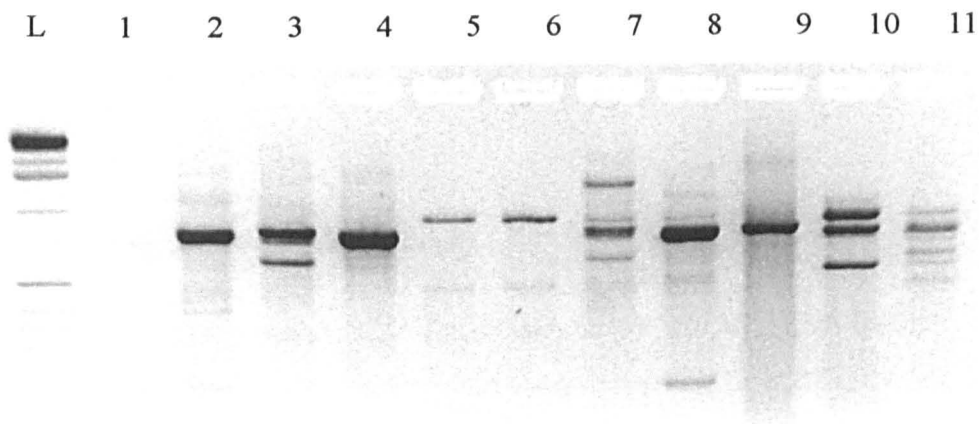


Figure 29. RISA of: 1: negative control, 2-6: 5 isolates from Gummern, 7: Nycodenz recovery from Gummern, 8-10: 3 isolates from Vermont, 11: Nycodenz recovery from Vermont.

Again, the banding patterns for the various species are very different and distinct. From first appearances, it seems that isolate 1 from Gummern (lane 2) and isolate 1 from Vermont (lane 8) are closely related with very similar profiles. There are also extra bands on the Nycodenz recovered samples (lanes 7 and 11) that are not present in any of the isolates, suggesting the presence of unculturable bacteria in the slurry. However, there are also bands present in the isolate profiles that are not in the Nycodenz preparations. This is because the number of rDNA operons expressed depends upon the growth state of the organism, so bacteria growing on nutrient rich agar plates are expressing more operons than those that are experiencing a degree of starvation in the poorer nutrient slurry. This finding makes it difficult to compare the slurry and isolates from the slurry directly, although culturing isolates on nutritionally poor media (i.e. Slurry Agar) may provide more comparable data.

The main limitation of this technique derives from the random patterns of variation of the spacer size and their unpredictability. Problems exist, such as growth rate altering operon expression, and closely related species having different spacers and the reverse. However, sequence information used in addition to the operon size patterns solves most of these problems. Overall, this technique is very powerful and versatile and is certain to go on yielding valuable information for the slurry community. Some of the bias problems experienced so far may be countered by experimenting

with reverse primers with better sequence conservation, but other bias problems are by the very nature of the technique unavoidable, and should be carefully considered in future work.

3.4 Effectiveness of Preservatives / Disinfectants

The major problem in using preservatives / disinfectants in the White Pigment Industry, but also in the paper and paint industry, is the lack of information concerning their effectiveness. The plants are permanently uncertain whether

- a) the preservatives / disinfectants are still showing bactericidal effects
- b) the quantity dosed is sufficient to eliminate viable cells or to prevent growth, respectively.

Due to the long incubation time as well as the selectivity of traditional analytical methods it is often not possible to make a statement before the product is delivered to the customer. However, this information which is very valuable to the plants can now be generated by the use of the CellFacts II technology.

In a study performed in the plant at Avenza (Italy) it was possible to show that the CellFacts II technology gives the possibility of real time preservative control, allowing the user to make a statement about the effectiveness of his preservative as well as to optimise the dosing and costs.

Samples were taken from Silo 1 and Silo 6, both treated with Biocide AK, then stained with DISC<sub>3</sub> and SYTO 62 and measured with CellFacts II (Table 14).

Sample	DiSC3		SYTO 62	
	TVC (cfu ml <sup>-1</sup> )	Average Fluorescence	TVC (cfu ml <sup>-1</sup> )	Average Fluorescence
Silo 1	1.1 x 10 <sup>6</sup>	54.59	1.4 x 10 <sup>6</sup>	27.7
Silo 6	-	-	9.9 x 10 <sup>2</sup>	6.01

Table 14. TVC in Silo 1 and 6 ex Avenza, measured using the CellFacts II technology.



Silo 1 returned a total viable count of  $> 10^6$  cfu ml<sup>-1</sup> with an average particle fluorescence of  $> 54$  for DiSC<sub>3</sub> and  $> 27$  for SYTO 62. These data show that Silo 1 has a high concentration of viable bacteria per ml.

Silo 6 returned TVC and average fluorescence counts that are indicative of a sample with  $< 10^3$  bacterial cells per ml. The results could be retraced since the biocide addition was made a few hours before the samples were taken and the full distribution might not have been guaranteed.

A detailed breakdown of the raw data from which the above were derived is given below:

Impedance profiles : Plot of particle size (equivalent spherical diameter) against particle count per ml (Figure 30).

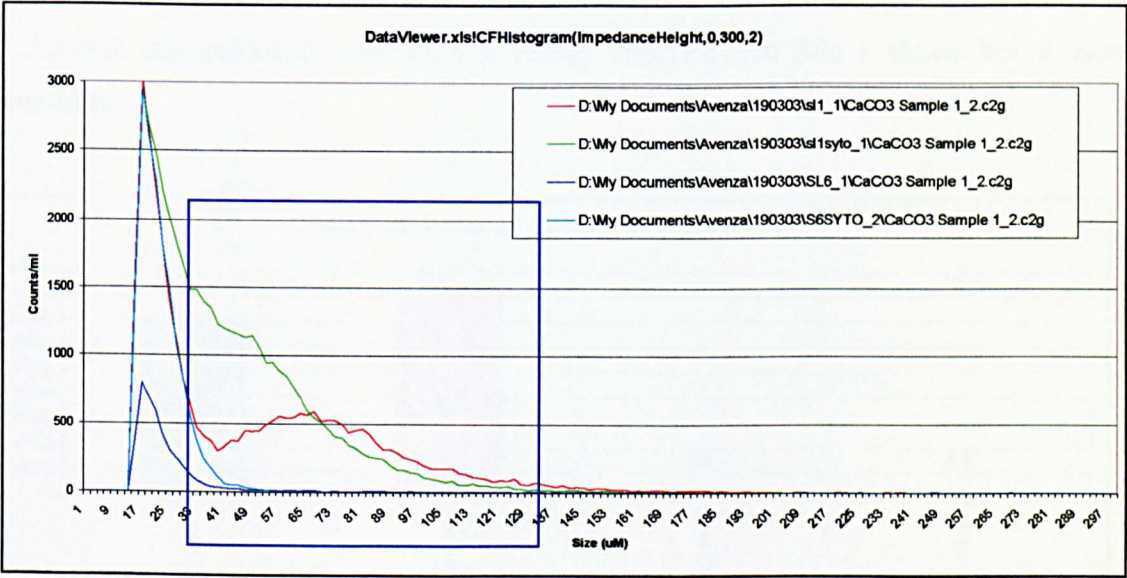


Figure 30. CellFacts II plot of particle size against particle count per ml. The box is indicative of the bacterial size range. Note: the x-axis which is representative of cell size is given in channel numbers.

From these data it is readily apparent that Silo 1 contains a high concentration of particles in the bacterial size range (boxed region) / ( — sample stained with DiSC<sub>3</sub>; — sample stained with SYTO 62).

Silo 6 displays profiles of background slurry particles only ( — sample stained with DiSC<sub>3</sub>; — sample stained with SYTO 62).

Based on these data it is not possible to comment on the physiological characteristics of this particulate population, i.e. effectiveness of the preservative. This would require analysis of the fluorescence signal from the two dyes, i.e. the above data are similar to those which would be generated by CellFacts I.

Number of Fluorescent Particles per ml

Histogram of the number of fluorescent particles per ml for samples from Silos 1 and 6 (Figure 31). As with the impedance profiles it is readily apparent that Silo 1 shows heavy bacterial contamination.

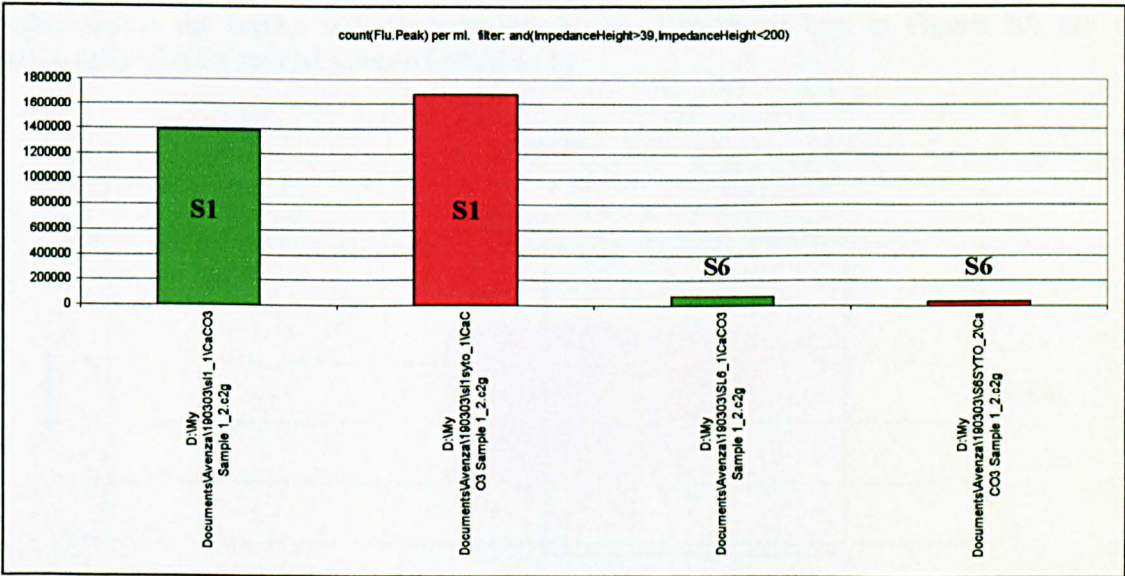


Figure 31. (■) DiSC<sub>3</sub> and (■) SYTO 62 staining. The y-axis = the number of fluorescent particles per ml from channel number 39 to 200 i.e. the bacterial size range.



Since SYTO 62 stains all cells, both viable and dead, whilst DiSC<sub>3</sub> stains only viable cells, the difference between the two analyses for silo 1 represents the number of dead cells present in the sample.

The fluorescence obtained from silo 6 shows that there are  $< 10^3$  bacteria per ml in these samples. The above is confirmed when scatter plots of the fluorescence values are examined.

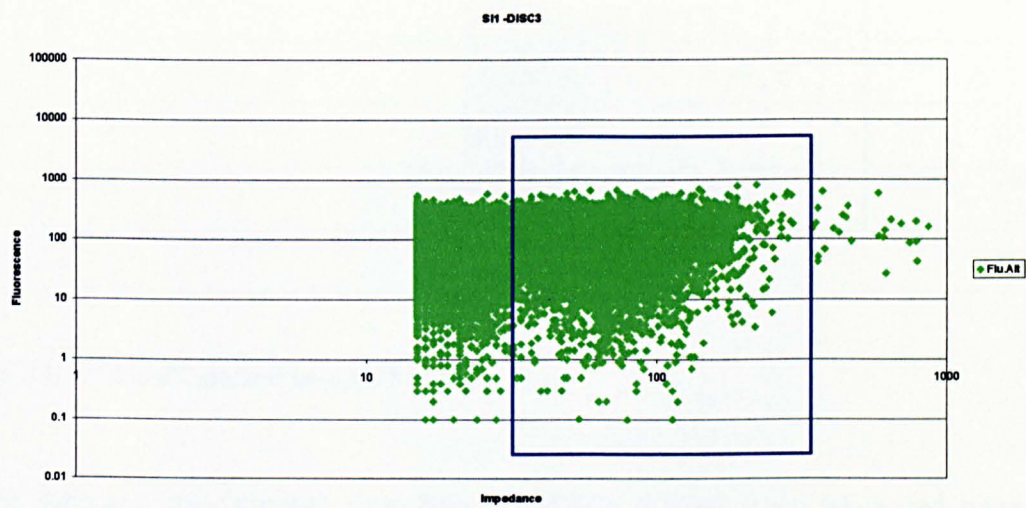


Figure 32. Plots of fluorescence intensity against particle size (impedance). The fluorescent particles within the boxed area (corresponds to the impedance box in Figure 30) are viable bacterial cells (DiSC<sub>3</sub> stained sample from Silo 1).

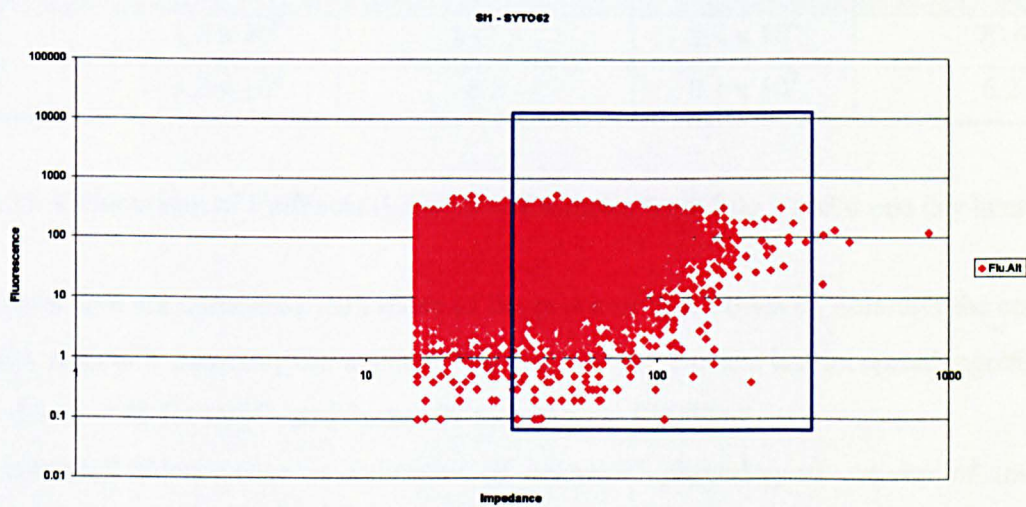


Figure 33. SYTO 62 stained sample from Silo 1. The fluorescent particles within the boxed area are both, viable and dead bacterial cells.

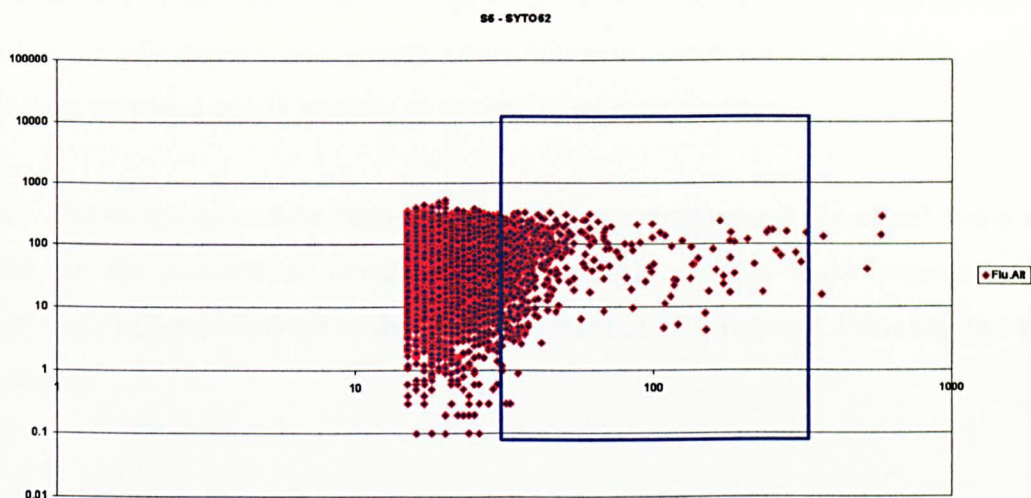


Figure 34. SYTO 62 stained sample from Silo 6.

On the following day, samples from Silo 1 and Silo 6 were again taken and measured using CellFacts II.

Sample	DiSC3		SYTO 62	
	TVC (cfu ml <sup>-1</sup> )	Average Fluorescence	TVC (cfu ml <sup>-1</sup> )	Average Fluorescence
Silo 1	1.6 x 10 <sup>6</sup>	137.3	2.4 x 10 <sup>6</sup>	70.46
Silo 6	1.2 x 10 <sup>3</sup>	8.2	8.1 x 10 <sup>2</sup>	6.24

Table 15. Comparison of CellFacts II analysis of samples from Silo 1 and 6 one day later.

The above data are consistent with those of the previous day, however, although the count per ml data has remained constant, the average fluorescence per particle has increased significantly, i.e. from ~54 to ~138 for DiSC<sub>3</sub> and from ~ 27 to ~70 from SYTO 62.

This increased fluorescence is indicative of enhanced physiological activity of the bacterial population – reduction in the activity/concentration of the biocide. In this case, the addition of Biocide AK was not sufficient to achieve a bactericidal effect.

This procedure enables a production site to determine the microbiological activity or a reduction in the concentration of the biocide in a storage tank nearly in real-time. This information on the bactericidal action of a preservative enables a) additional preservation to be carried out quickly and thus costs to be saved and b) minimizing the risk of bacterial resistance.

A fact which should not be underestimated is that this procedure reveals the actual situation in a storage tank, i.e. the presence of dormant or compromised cells that could "regenerate" when sufficient nutrients become available or the biocide concentration is reduced. False negative results will be prevented.

### 3.5 Stability of Preservation

Stability of preservation is extremely important. On the one hand, the preservative is expected to produce a rapid microbicidal effect for successful control of contamination that has already occurred, on the other hand it has to show a certain long-term stability to prevent subsequent bacterial contamination / growth.

Therefore, a study was carried out to investigate the behaviour of a  $\text{CaCO}_3$  slurry from Verpol (USA) which had been preserved using three different types of preservatives and transported by rail waggons to three different customers. For this purpose, the samples were inoculated with  $5.0 \times 10^4$  bacteria per ml, isolated from  $\text{CaCO}_3$  slurry ex Verpol. After the subsequent storage of the samples for 24 hours at  $30^\circ\text{C}$ , the total viable count was determined by means of CellFacts II. Additional inoculations were made until the total viable count of the samples was  $> 10^4$  cfu ml<sup>-1</sup> (Table 16). The number of possible additional inoculations without viable contamination gave an indication of the quality of the preservative action in the product. These results showed that significant differences were found in the analysed samples. In this study, the  $\text{CaCO}_3$  slurry treated with OPP was identified as having the best preservative properties.



Preservative	TVC before Inoculation	Number of Inoculations
2-methyl-4- isothiazoline-3-one / 5-chloro-2-methyl-4-isothiazoline-3-one	200 cfu ml <sup>-1</sup>	1
3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione	< 100 cfu ml <sup>-1</sup>	3
o-Phenylphenol	< 100 cfu ml <sup>-1</sup>	5

Table 16. Stability of preservation of 3 CaCO<sub>3</sub> slurries ex Verpol.

Figure 35 shows the CaCO<sub>3</sub> slurry treated with 2-methyl-4-isothiazoline-3-one / 5-chloro-2-methyl-4-isothiazoline-3-one, before (violet) and after (blue) the inoculation was approx.  $5.0 \times 10^4$  cells per ml. The initial total viable count measured was  $2.0 \times 10^2$  cfu ml<sup>-1</sup>. After the inoculation and incubation for 24 hours at 30°C, however, the total viable count was  $2.6 \times 10^6$  cfu ml<sup>-1</sup>. The residue of the biocide in the sample was not sufficient to prevent subsequent growth of the viable microbial population. This is not surprising since the blend of 2-methyl-4-isothiazoline-3-one with 5-chloro-2-methyl-4-isothiazoline-3-one is known as a fast killer with short-term stability.

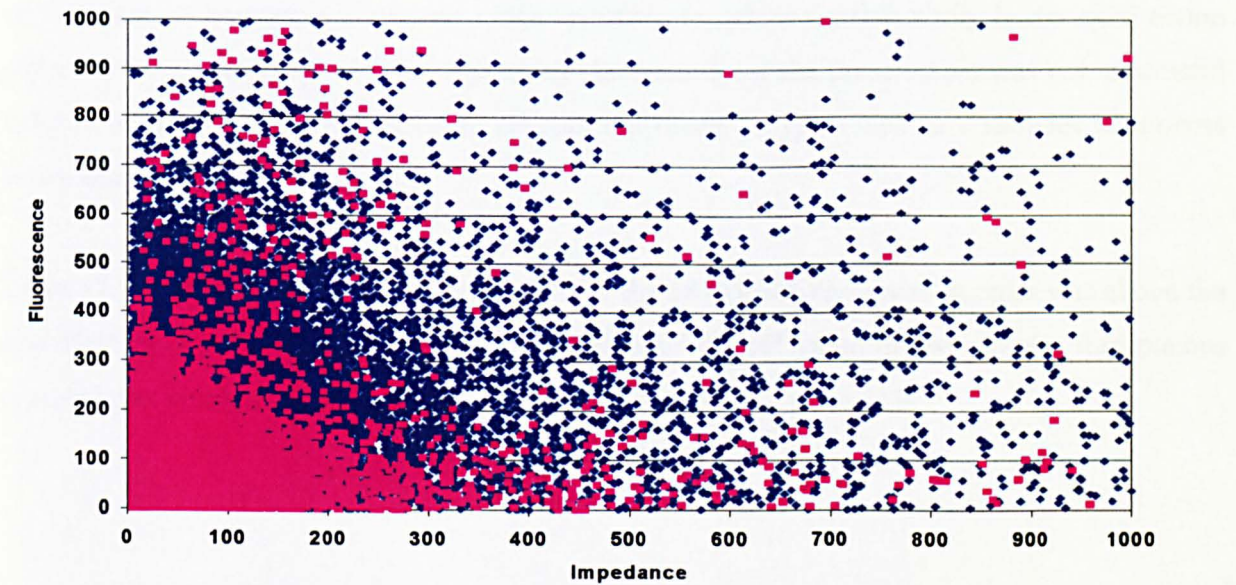


Figure 35. CellFactors II plot showing the instability of isothiazolines. Here we have a scatter plot of a contaminated slurry treated with isothiazolines showing bactericidal inefficiency with incubation. (pink = effective, dark blue = ineffective bactericidal action).

The excellent long-term stability of o-phenylphenol was confirmed in further studies carried out in other plants, which makes this active agent interesting for the preservation of White Pigment Slurries.

### 3.5.1 Resistance

The problem of resistance of microorganisms to preservatives and disinfectants is an increasingly important subject for the White Pigment Industry as well as for their customers. As discussed in more detail in sections 4.5 and 5.3.1, resistances can have serious consequences for the use of antimicrobials, both from a technical as well as a commercial point of view. On the one hand, the choice of biocides is becoming more and more constrained by new regulations (i.e. BPD), on the other hand, resistance is becoming more relevant due to the lack of continuous monitoring or house-keeping for cost reasons. The following example clearly illustrates that the detection and understanding of resistance is extremely important if appropriate alternatives are to be identified or possibilities to solve the problem devised.

In the plant at Gummern it was no longer possible to achieve satisfactorily bactericidal action using the usual quantities of OPP. Increasing the quantity of the preservative was not successful either. For this reason, 38 samples of calcium carbonate slurry as well as 2 samples of process water were analysed.

Table 17 shows, that the bacterial count of 11 of the 38 calcium carbonate samples was above the specification of  $> 10^3$  cfu ml<sup>-1</sup>. The determination of the pH value of the samples furthermore revealed that nearly all samples showing bacterial growth had a pH of  $< 9$ .

Product	Tank	TVC (cfu ml <sup>-1</sup> )	PH
H60 78% (15.08.02)	T 1000/4	<100	9.0
H60 78% (16.08.02)	T 1000/4	100	9.3
H60 78% (29.08.02)	T 1000/4	<100	9.0
H60 78% (03.09.02)	T 1000/4	<100	9.2
H60 78% (26.09.02)	T 1000/4	<100	-
H60 78% (17.08.02)	T 1000/8	1.0 x 10 <sup>5</sup>	8.3
H60 78% (20.08.02)	T 1000/8	>10 <sup>5</sup>	8.4
H60 78% (29.08.02)	T 1000/8	<100	9.1
H60 78% (04.09.02)	T 1000/8	<100	9.6
H60 78% (26.09.02)	T 1000/8	<100	-
H60 78% (05.09.02)	Customer tank <sup>1)</sup>	>10 <sup>5</sup>	8.6
H60 65% (19.08.02)	T 1000/9	<100	9.1
H60 65% (21.08.02)	RC 6418-2 top	>10 <sup>5</sup>	8.7
H60 65% (21.08.02)	RC 6418-2 bottom	2.0 x 10 <sup>5</sup>	8.5
H60 65% (23.08.02)	Customer tank <sup>2)</sup>	>10 <sup>5</sup>	8.4
H60 65% (29.08.02)	T 1000/9	100	9.7
H60 65% (26.09.02)	T 1000/9	<100	-
H60 65% (29.08.02)	T 2000/3	100	9.0
H60 65% (26.09.02)	T 2000/3	<100	-
H90 76.5% (02.09.02)	T 2000/2	>10 <sup>5</sup>	8.2
H90 76.5% (03.09.02)	T 2000/2	>10 <sup>5</sup>	8.3
H90 76.5% (05.09.02)	T 2000/2	<100	9.7
H90 76.5% (26.09.02)	T 2000/2	<100	-
H90 76.5% (26.09.02)	T 2000/2	>10 <sup>5</sup>	-
H90 76.5% (01.10.02)	T 2000/2	<100	9.3
H90 76.5% (04.10.02)	T 2000/2	<100	9.2
H90W 76.5% (26.09.02)	T 1000/3	<100	9.4
H90 78% (26.09.02)	T 2000/4	<100	-
H70 75% (26.09.02)	1400	<100	-
CC75 71.5% (26.09.02)	2000/5	6.0 x 10 <sup>3</sup>	-
CC75 71.5% (11.10.02)	2000/5	>10 <sup>4</sup>	8.8
CC75 71.5% (11.10.02) <sup>3)</sup>	2000/5	<100	9.2
CC60W 72% (26.09.02)	2000/1	200	-
Set-HG (26.09.02)	T 1000/6	<100	-
Set-HG (26.09.02)	T 1000/7	5.0 x 10 <sup>4</sup>	-
Set 78% (26.09.02)	T 1000/5	<100	-
Set 78% (26.09.02)	500 (sieve tank)	600	-
Set 75% (26.09.02)	T 1000/2	<100	-

<sup>1)</sup> storage tank customer A

<sup>2)</sup> storage tank customer B

<sup>3)</sup> Samples were taken and analysed 3 days later, stored for another 32 days and measured again.

Table 17. TVC and pH measurement of 38 calcium carbonate slurries from Gummern.



In a further step, several of the samples were analysed for their biocide content (OPP). The analyses showed that the biocide dose in the samples was in the lower range (Table 18). It has however to be considered here that some of the samples were quite old. Furthermore, it was observed that bacterial growth was also found in samples with high biocide content. From these results, the minimum inhibition concentration of OPP was determined (Table 19).

Sample	ppm OPP <sup>1)</sup> (Recommended Volume)
T 1000/4 (15.08.02)	-
T 1000/4 (16.08.02)	156 (190)
T 1000/4 (29.08.02)	160 (190)
T 1000/4 (03.09.02)	-
T 1000/8 (17.08.02)	-
T 1000/8 (20.08.02)	171 (190)
T 1000/8 (29.08.02)	280 (190)
T 1000/8 (04.09.02)	-
Customer <sup>2)</sup> (05.09.02)	-
T 1000/9 (19.08.02)	311 (355)
RC 6418-2 (21.08.02)	295 (355)
RC 6418-2 (21.08.02)	319 (355)
Customer <sup>3)</sup> (23.08.02)	-
T 1000/9 (29.08.02)	275 (355)
T 2000/3 (29.08.02)	319 (355)
T 2000/2 (02.09.02)	160 (202)
T 2000/2 (03.09.02)	161 (202)
T 2000/2 (05.09.02)	239 (202)
T 2000/1 CC60W 72%	333 (255)
T 2000/5 CC75 71.5%	289 (250)
T 1000/7 Set HG 74%	176 (230)
T 1000/5 Set 78%	169 (190)
T 500 Set 78%	169 (190)

<sup>1)</sup> ppm active OPP per dmt calcium carbonate slurry

<sup>2)</sup> rail car at load out customer A

<sup>3)</sup> storage tank customer B

Table 18. OPP analysis of a selection of calcium carbonate slurry samples from Gummern.

HC90-GU 75%	TVC (cfu ml <sup>-1</sup> )			
	Control	H60 78% (20.08.02) <sup>1)</sup>		GU-Standard <sup>2)</sup>
		220ppm OPP <sup>3)</sup>	300ppm OPP <sup>3)</sup>	220ppm OPP <sup>3)</sup>
1 <sup>st</sup> Inoculation	>10 <sup>5</sup>	>10 <sup>5</sup>	2.0 x 10 <sup>4</sup>	<100
2 <sup>nd</sup> Inoculation	>10 <sup>5</sup>	>10 <sup>5</sup>	—	<100
3 <sup>rd</sup> Inoculation	>10 <sup>5</sup>	—	—	<100

<sup>1)</sup> The samples were inoculated with contaminated 78% dd. 20.08.02.  
<sup>2)</sup> The samples were inoculated with our "standard microbes" from Gummern.  
<sup>3)</sup> Preventol OF45: active/solids content

Table 19. MIC study for the verification of the bactericidal performance of Preventol OF 45.

Table 19 shows that the "new" microbes were able to grow despite the addition of OPP whereas the "old" standard microbes were eradicated with the usually recommended quantity of OPP. Due to these results, it cannot be ruled out that new species which are resistant to OPP have been selected / enriched or resident species have adapted to the presence of OPP.

In a further step, the different bacteria were isolated and spread onto Plate Count Agar containing 1000 ppm OPP (active). Figure 36 shows that some species have adapted to the quantities of OPP used or were not destroyed by the preservative.

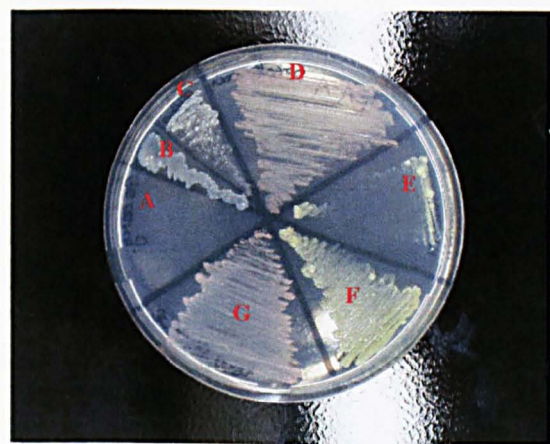


Figure 36. PCA plate containing 1000 ppm active OPP.

- A) *Ps. aeruginosa* (sensitive)
- B) white pigmented species (not sensitive)
- C) white pigmented species (not sensitive)
- D) pink pigmented species (not sensitive)
- E) yellow pigmented species (not sensitive)
- F) yellow pigmented species (not sensitive)
- G) pink pigmented species (not sensitive)

As a medium-term measure, due to the results described above, different biocides for the subsequent preservation of the Gummern slurries were examined (Table 20). These experiments showed that all of these active agents were able to eradicate the microbes that were present.

Source of Bacteria for the Inoculation of H90 75%	TVC (cfu ml <sup>-1</sup> )		TVC (cfu ml <sup>-1</sup> )			
	Control	250OPP	+250 AK <sup>2)</sup>	+250 BK <sup>3)</sup>	+100 Glut <sup>4)</sup>	+ 50 THPS <sup>5)</sup>
Gummern Standard	>10 <sup>5</sup>	<100	—	—	—	—
Process water 11.10.02	—	>10 <sup>5</sup>	—	—	—	>10 <sup>4</sup> <sup>1)</sup>
CC75 71.5% 11.10.02	>10 <sup>5</sup>	>10 <sup>5</sup>	<100	—	—	—
Set HG 26.09.02	—	>10 <sup>5</sup>	<100	—	—	—
1) H60 78% 20.08.02	>10 <sup>5</sup>	>10 <sup>5</sup>	—	<100	—	—
2) H60 78% 20.08.02	>10 <sup>5</sup>	>10 <sup>5</sup>	—	—	<100	—
3) H60 78% 20.08.02	>10 <sup>5</sup>	>10 <sup>5</sup>	—	—	—	<100

<sup>1)</sup> The test was repeated using 100 ppm THPS. TVC < 100 cfu ml<sup>-1</sup>

<sup>2)</sup> Biocide AK per dmt CaCO<sub>3</sub>

<sup>3)</sup> Biocide BK14 per dmt CaCO<sub>3</sub>

<sup>4)</sup> Glutaraldehyde (active) per dmt CaCO<sub>3</sub>

<sup>5)</sup> THPS (active) per dmt CaCO<sub>3</sub>

Table 20. Performance of different microbiocides to bacteria showing resistance to OPP.

In addition to the slurry samples, the process and wash water were analysed. Both showed high bacterial contamination. Furthermore, anaerobic growth was detected in both water samples. In a further experiment, these microbes were used for the inoculation of slurry samples preserved using OPP. It was found that these organisms (in slurry) were also able to grow although they had been previously inhibited by OPP (Table 21).

H90-GU 75%	TVC (cfu ml <sup>-1</sup> )	
	Control	250ppm OPP <sup>1)</sup>
Gummern Standard	>10 <sup>5</sup>	<100
Wash water 26.09.02	>10 <sup>5</sup>	>10 <sup>4</sup>
Process water 26.09.02	>10 <sup>5</sup>	2.0 x 10 <sup>4</sup>

<sup>1)</sup> Preventol OF45: active per dmt CaCO<sub>3</sub> slurry – calculated on 75 % solids

Table 21. Sensitivity study of OPP to bacteria from wash and process water.

Since each of the active agents tested before were able to eliminate these organisms, too, it can be assumed that the two waters have contributed to the introduction of resistant species. The process water runs from the PX into production whereas the wash water is used for cleaning the rail waggons.

In a last experiment, the pH of the contaminated water was adjusted to a value of 12 to test whether the existing microbes could be destroyed in this way. Table 22 shows that, in both cases bacterial growth was strongly reduced after 24 hours incubation.

	TVC (cfu ml <sup>-1</sup> )			
	0h <sup>1)</sup>	1h <sup>1)</sup>	2h <sup>1)</sup>	24h <sup>1)</sup>
Wash water (pH 12)	>10 <sup>3</sup>	>100	>100	<10
Process water (pH 12)	>10 <sup>4</sup>	>10 <sup>3</sup>	>10 <sup>3</sup>	30

<sup>1)</sup> pH was adjusted with NaOH to 12. TVC was determined after 0, 1, 2 and 24 hours.

Table 22. Elimination of bacterial growth under strong alkaline conditions.

Furthermore, a residual OPP concentration of 2 - 3 ppm was detected in both water samples. This is not surprising since the increasing recirculation of process water and other resources into the production process, i.e. the increasing trend towards using closed circuits within the production sites, leads to low concentrations of biocides being recirculated into the system. This involves the risk of a familiarization and, eventually, enhancement of the resistance of the microorganisms to the biocidal active agents or selection of resistant species.

Furthermore, it has to be remembered that closed circuits provide microbes with conditions which are quite comparable to those of a fermenter. This means that the introduction of a stringent house-keeping system (chapter 5) is inevitable.

In this case, as a remedial action, the disinfection of both the process and wash water had to be considered. In the long-term, an "alkaline" disinfection would offer a valuable "low-cost" alternative.

### 3.6 Control of Microbial Activity - QC Methods

Prevention of contamination caused by microorganisms and the effects arising therefrom have significant economic consequences. These consequences must not be underestimated. For example, significant changes in the rheology of mineral dispersions can lead to a standstill in paper production. Similarly, continual false application of biocide(s) arising from an over-reaction philosophy can have equally dramatic economical effects.

To achieve the goal of optimal control of microbial contamination continuous monitoring of the biological activity in a system and well-balanced housekeeping (i.e. storage tank and transport cleanliness) are necessary. Together with monitoring, the process of optimal dosing and selection of biocide(s) becomes an integrated part of the strategy. It is therefore necessary to consider carefully the options available both currently and those under development for isolation and monitoring of bacteria, as rapid identification and concentration are crucial to the benefits that can be derived from optimising the control strategy.

Over the last years, classic methods for the determination of the total viable count have become established in the white mineral industry. The use of agar substrates in a Petri dish, or on a plastic film, are primarily suitable for a first count (and isolation) of aerobic or facultatively anaerobic microorganisms. This method can also be applied to anaerobes provided the plates are incubated in an anaerobic jar. A significant disadvantage of the plate method for determining the total viable count is the long incubation times (culture enrichment) required (typically 48-72 hours) which strongly impairs the ability to apply quality control, and hinders online production security. Figure 37 clearly shows that, due to the long incubation times of traditional methods, the results do not reflect the current situation in the storage tank but the situation as it was some ~ 48 hours before.



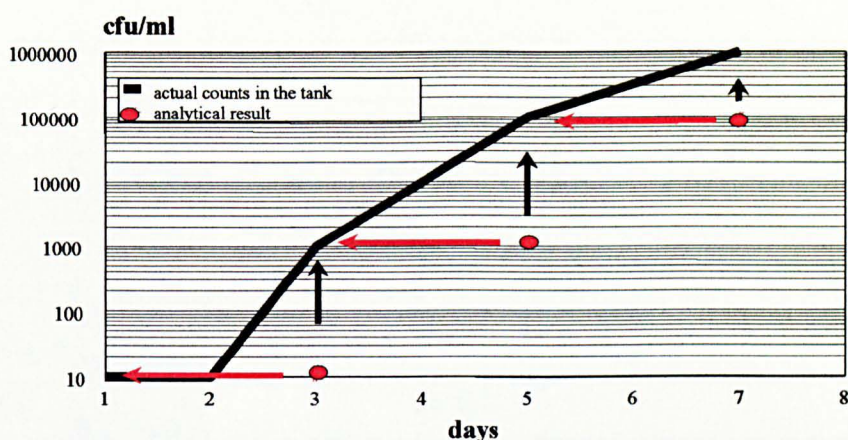


Figure 37. Disadvantage of long incubation times when applying traditional methods. The actual counts in a storage tank are never reflected.

New applications of established instrumental techniques, such as electronic cell counting, based on flow impedance, as well as methods for vital counting (vital staining), offer interesting alternatives to the agar substrates. These options are reviewed in the following sections. Furthermore, we go on to consider novel methods of electro-optically identifying the bacteria undergoing vital staining.

### 3.6.1 Plate Count

As mentioned above significant disadvantages of traditional plating methods for determining the total viable count are the long incubation time and the selectivity (it is estimated that less than 1% of the total number of species present in naturally occurring soil/sediment samples are culturable) which strongly impairs the ability to apply quality control, and hinders online production security.

The following results (Table 23) were generated at the production site in Norway and clearly show the difficulty in obtaining information about the physiology of the bacterial cells.

Samples of a fine calcium carbonate slurry were taken at different points in production. Table 23 lists the temperatures of each sample when it was taken and the total viable count of the aerobic bacteria in the product.



Sample	Temp. (°C)	TVC (cfu ml <sup>-1</sup> )
1) PX feed	53°C	<100
2) PX water <sup>1)</sup>	80°C	<100
3) after PX8	85-90°C	<100
4) after PXs	73-81°C	<100
5) before evaporator	75-80°C	<100
6) after evaporator	49-57°C	<100
7) before tank <sup>2)</sup>	54°C	<100
8) Tank 1817	46°C	<100
9) Loadout: 1816 <sup>1)</sup>	—	<100
10) Thickener underflow <sup>2)</sup>	40°C	>10 <sup>4</sup>
11) Tank 1802 <sup>2)</sup>	39°C	10 <sup>4</sup>
12) Tank 1803 <sup>1)</sup>	39°C	10 <sup>4</sup>
13) low level Tank 1822 <sup>1)</sup>	—	10 <sup>3</sup>
14) low level Tank 1823 <sup>1)</sup>	—	>10 <sup>4</sup>

Samples were taken on 07/08 & 09 May 02. The numbers shown are the mean of two or three measurements, unless the differences were large. In some cases (indicated) only one or two samples were taken.

<sup>1)</sup> One sample was taken.

<sup>2)</sup> Two samples were taken.

Table 23. TVC and temperature of calcium carbonate slurry samples taken at the production site in Norway.

According to the results generated it can be assumed that the calcium carbonate slurry product is very clean. No bacterial growth was found at any point after the PXs (samples 1 - 9). Even the PX feed was clean (< 100 cfu ml<sup>-1</sup>). This could be due to the fairly high (> 50°C) temperatures during the production.

However, two aspects have to be discussed here:

- It is possible that the microorganisms are destroyed by the high temperature and that they are dead or in a physiologically stressed or compromised state. Such a state would hardly be (or even not) detected using plate count methods. However, information on the presence of such cells is of importance since the performance of preservatives can be negatively affected.
- Since the temperatures during biocide addition are generally also quite high (sample 7), it cannot be ruled out that the efficiency of the preservative is negatively affected due to the lack of thermal stability (section 4.4). Here again, the lack of information about the physiology of the bacterial cells has to be considered.

Evidence for the hypothesis above is provided by the following study:

A sample of a calcium carbonate slurry was taken during the loadout of the vessel. The sample was from a storage tank into which no product had been filled for 4 days. At the time of the first measurement of the bacterial count (3 days after the sample had been taken) no bacterial contamination was found. The determination of the biocide content showed that the product had originally been preserved with 557 ppm Biocide ABK (commodity/dry), but no active components were subsequently found. This means that, although the product was clean on loading, it was not sufficiently preserved. This was confirmed in the laboratory. 10 days later (storage at room temperature), the bacterial count of the sample was  $> 10^4$  cfu ml<sup>-1</sup>.

Due to the lack of information about the physiology of bacterial cells within the product with the Plate Count method it was not possible to verify if the sample was at risk or not. Here clearly a high number of physiologically compromised cells passed the PX. The biocide then was "used up" in order to keep the cells in this status or to even kill them but was not able to maintain a preservative effect.

### 3.6.2 CellFacts I

The primary aim was to find a quick method for determining the TVC within White Pigment Slurries. CellFacts I, which is based on the principle of electrical flow impedance (Section 2.3.5) offered a solution.

The time required for the analysis can significantly be reduced, but verification of the physiological state of the cells is not possible. Furthermore, it has to be remembered here that, due to the use of nutrient media, growth will be selective.

#### 3.6.2.1 Sample Preparation

Due to the high number of particles  $> 10^{10} \text{ ml}^{-1}$  in a White Pigment Slurry it is not possible to analyse the samples directly using the measuring system. Bacterial counts in the range of approx.  $10^3 - 10^7 \text{ cfu ml}^{-1}$  would disappear in the background noise of the apparatus. Furthermore, dilution of the sample would, of course, also massively compromise the detection limit of the TVC. For this reason, separation of the pigment particles by means of a centrifugal step is necessary.

In a study carried out on site in Perth (Canada) the sample preparation was optimised so that it was easily applicable in the daily quality control at a production site.

Experiments using calcium carbonate slurry samples indicated that, using a fixed angle rotor, 2,000 g for 10 minutes (g value conversion not available) were the most suitable settings when 4 ml slurry was added to 8 ml of tryptic soy broth (TSB) and homogenised. After centrifugation the supernatant was recovered and incubated at 37°C. The samples were then analysed via CellFacts I at  $t_0$  and at defined time intervals thereafter (Figure 39).

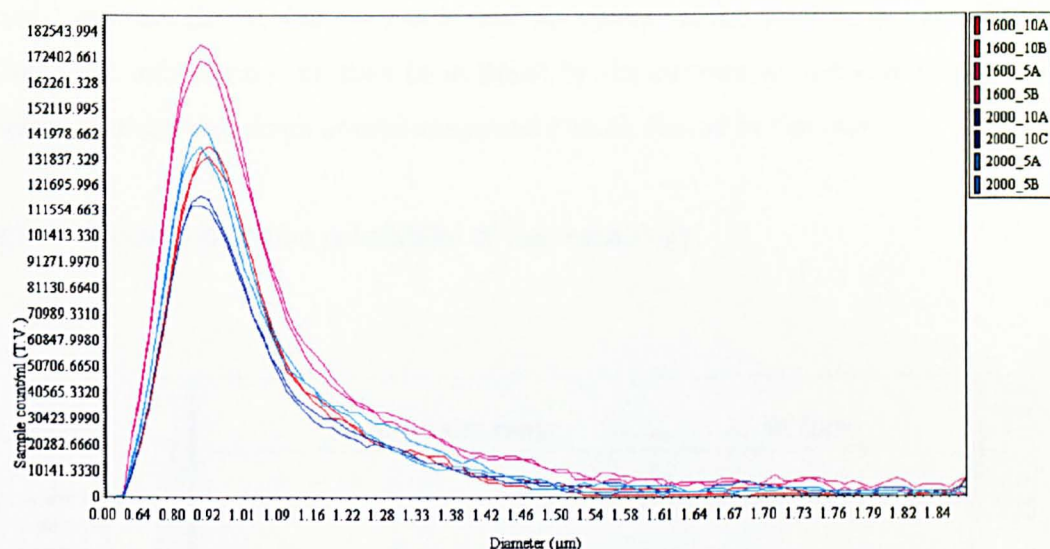


Figure 38. Particle size distribution profiles (in duplicate) from the supernatant of a calcium carbonate slurry sample centrifuged at 1,600 or 2,000 g for 5 or 10 minutes.

Samples were prepared as per the protocol but centrifuged at 1,600 and 2,000 g for 5 or 10 minutes. As would be expected, increased speed and time reduced the particle load in the supernatant. From the profile data centrifugation of all subsequent samples was standardised on 2,000 g for 10 minutes – suitable balance between removing the  $\text{CaCO}_3$  particles but retaining the bacterial cells in the supernatant.

Clearly there are questions over the sample preparative technique – particularly the centrifugation step. When optimised, and performed without deviation from the set procedure, the percentage recovery of bacterial cells from a particular  $\text{CaCO}_3$  sample is reproducible, but recovery varies between different  $\text{CaCO}_3$  sample types. The procedure is operator dependent. Also of importance are the g values generated by the centrifuge – for bench top centrifuges these may vary significantly from one centrifuge to the other. Ideally the centrifuge being used should be optimised with respect to bacterial recovery from both coarse and fine  $\text{CaCO}_3$  samples. A more satisfactory solution would be the implementation of the “new” preparative procedure devised for CellFacts II (Nycodenz technique – section 3.6.3.1). This minimises sample and operator error.

3.6.2.2 Evaluation of Results

CellFacts I provides the number of particles and the volume of the particles in a sample, off- or on-line. This basic information can then be analysed by the customised software to provide precise, real-time data in the size-range or time-range and format desired by the user.

Figures 39-41 clearly show the possibilities of this technology:

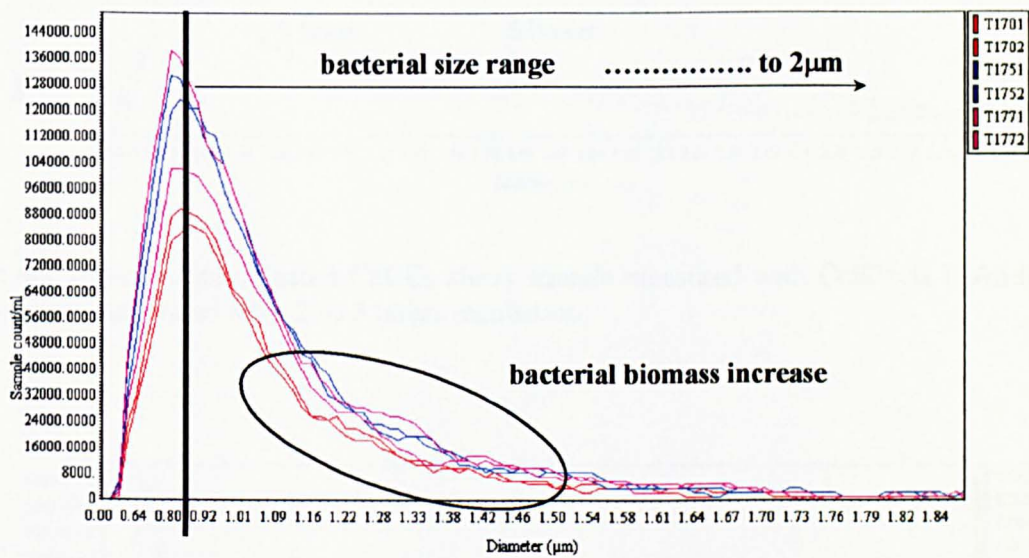


Figure 39. CaCO<sub>3</sub> slurry samples after t0, t5 and t7 hours incubation. There is a marginal increase in particle i.e. bacterial load from t0 to t5 but this is not so obvious in the t7 sample.



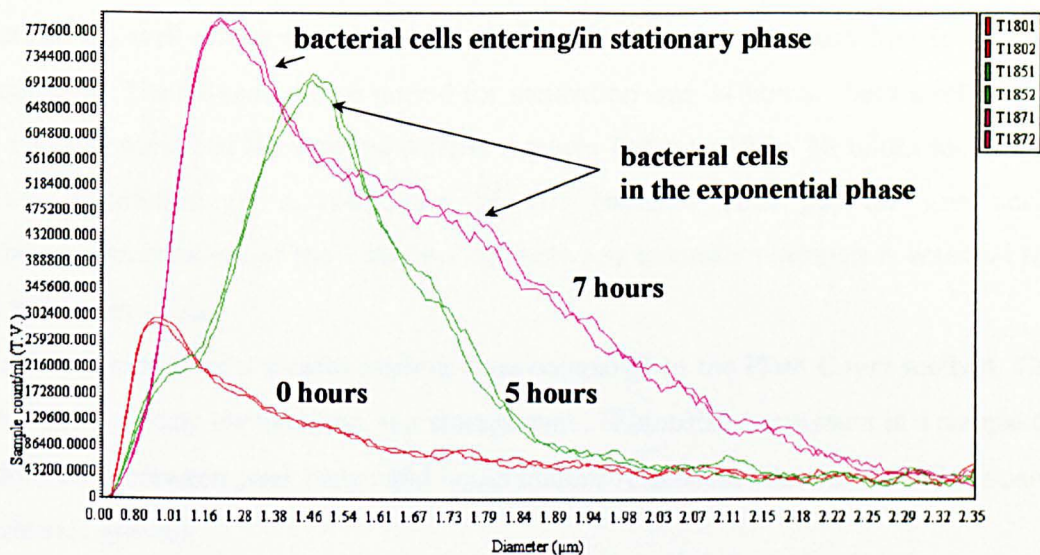


Figure 40. Heavily contaminated  $\text{CaCO}_3$  slurry sample measured with CellFacts I. An instrument response was generated after 2 to 3 hours incubation.

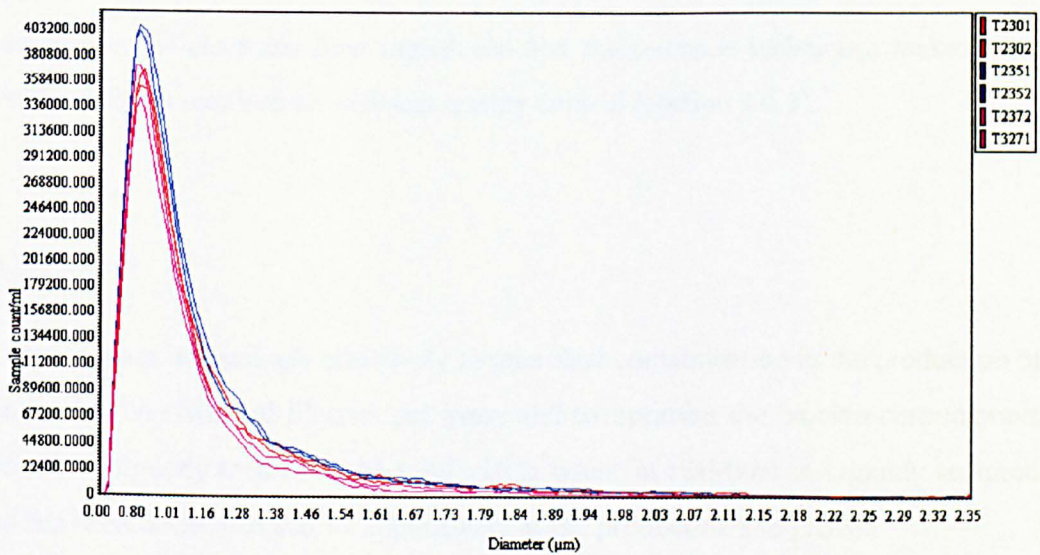


Figure 41. CellFacts I profile of a "clean"  $\text{CaCO}_3$  slurry. No significant change over the incubation time course.



The large number of analyses carried out with the Cellfacts I technology clearly showed that 48 hours is a too long an incubation time for White Pigment Slurry samples as the bacterial population was well into the stationary phase even for samples with very low levels of bacterial contamination. The maximum time period for incubation was 24 hours. Low level contamination i.e. < 10 cells per ml in the original sample requires between 15 to 18 hours for a result to be recorded. Resolution to a breakpoint of  $10^3$  bacterial cells per ml was achieved in 2 - 8 hours. Optimisation of the time intervals between successive samples is essential to achieve the sensitivity required.

Beside the advantage of a shorter analysis time compared to the Plate Count method, CellFacts I reflected more closely the situation in a storage tank. If biocides are present in a sample there will be a difference between pour plates and liquid culture re biocide dilution and effectiveness which will influence growth.

However, to be able to react to microbial contamination of a production of ~12 Mio. tonnes White Pigment Slurry per year and to optimise the preservative costs a method fulfilling the measurement of direct total viable counts (all microorganisms) as well as its determination in real-time is required.

The combination of electrical flow impedance and fluorescence techniques makes it possible to achieve the analyses required for efficient quality control (section 3.6.3).

### 3.6.3 CellFacts II

To be able to react increasingly effectively to microbial contamination in the production of millions of tonnes of White Mineral Slurries per year, and to optimise the biocide consumption costs, a method which directly measures the total viable count in real-time is urgently required. Such a method has been developed and its applicability at the production site proven.

A combination of flow impedance and fluorescence techniques has been investigated and developed in application to White Mineral Slurries and is now seen to offer a potential solution.

### 3.6.3.1 Sample Preparation

A patented sample preparation technique is followed to allow the instrument to be used with high solid suspensions containing particulate fillers and pigments by extracting preferentially the microbial material. Clearly, the challenge in preparing the sample is the separation of the microorganisms from the matrix. To be able to detect just a few bacterial cells in a matrix with a high number of other "background" particles of occurrence ratio up to  $1.0 \times 10^{10}$ , separation is essential since the bacteria are effectively "lost" since the particle size of primary pigment particles are in the same range.

Initial sample preparation procedures consisted of dilution of White Pigment Slurries followed by direct centrifugation. The supernatant was then separated and used for analysis. The recovery of bacteria from the samples was typically ~ 10% of those suspected to be present (determined on PCA agar plates from pre- and post-spin samples), although this recovery can range from < 1% to complete recovery depending on the systems involved - depending especially on the types of dispersing technologies in use.

The newly developed sample preparation method, which was in another form originally used successfully to concentrate and isolate microbes from soil (Rickwood, 1982), relies upon a density gradient cushion to separate bacteria from the matrix during centrifugation. This technique essentially entails centrifugation of an aqueous system in the presence of a disruption buffer mixture which is pipetted onto the top of a layer of a density gradient cushion material ( $1.3 \text{ g ml}^{-1}$ ).

Following centrifugation, the supernatant which contains the upper aqueous phase and the "density gradient" solution are collected. Since bacteria are resolved at the interface between the two layers they will be present in the supernatant, while pigment particles can pass through the density gradient cushion and form a pellet at the bottom of the tube (Figure 42). The supernatant is easily separated by pipetting or decanting into a new sterile tube and can then be used for the analysis.

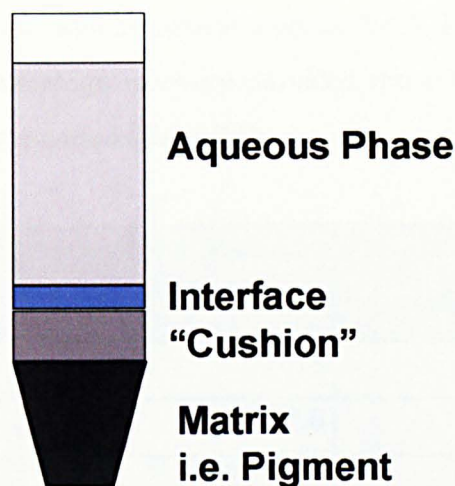


Figure 42. Separation of bacteria from the matrix by using a density gradient cushion (section 2.3.5).

In the course of the evaluation it became apparent that there were occasional differences between the plate culture cfu count for raw and extracted samples. These differences could only be attributed to either the loss of cells during the centrifugation steps or the effect of nycodenz on the cells.

Further experimentation then confirmed that few cells are lost as a consequence of centrifugation. However, the effect of nycodenz on cells is less clear. This is a polymeric derivative of benzoic acid and benzoic acid *per se* may have a bacteriostatic effect.

During protocol development the effect of nycodenz on a range of bacterial species isolated from  $\text{CaCO}_3$  slurries was investigated. It was established that nycodenz had little or no effect on viability. However, more recent studies have shown that late stationary phase cultures are more susceptible to nycodenz than exponential or early stationary phase cells. Nycodenz, at the concentration necessary to separate the bacterial cells from the  $\text{CaCO}_3$ , may cause lysis of a percentage of the cell population when cells are in the late stationary phase. This may also be the case when cells have been physiologically compromised by biocide.

In further experiments sucrose (60% (w/v)) was examined as an alternative density substrate to nycodenz. The major problem with sucrose is osmotic shock. However, this is time dependent and does not influence the percentage recovery provided the cells are not retained in the sucrose solution for an extended time period (Table 24).

Sample	Average Fluorescence	CellFacts II Count (cfu ml <sup>-1</sup> )	Plate Count (cfu ml <sup>-1</sup> )
No 1 - Nyc / DiSC <sub>3</sub>	22.1618	2.6 x 10 <sup>5</sup>	5.6 x 10 <sup>5</sup>
No 1 - Nyc / SYTO62	48.5298	8.7 x 10 <sup>5</sup>	
No 1 - Suc / DiSC <sub>3</sub>	24.8026	3.2 x 10 <sup>5</sup>	1.0 x 10 <sup>4</sup>
No 1 - Suc / SYTO62	38.8072	3.7 x 10 <sup>5</sup>	
No 2 - Nyc / DiSC <sub>3</sub>	28.5093	1.4 x 10 <sup>5</sup>	1.9 x 10 <sup>5</sup>
No 2 - Nyc / SYTO62	47.5297	5.1 x 10 <sup>5</sup>	
No 2 - Suc / DiSC <sub>3</sub>	28.0868	1.0 x 10 <sup>5</sup>	7.3 x 10 <sup>4</sup>
No 2 - Suc / SYTO62	43.2685	2.0 x 10 <sup>5</sup>	
No 3 - Nyc / DiSC <sub>3</sub>	34.6317	3.8 x 10 <sup>5</sup>	3.3 x 10 <sup>4</sup>
No 3 - Nyc / SYTO62	51.1990	3.2 x 10 <sup>5</sup>	
No 3 - Suc / DiSC <sub>3</sub>	33.7401	7.9 x 10 <sup>4</sup>	3.3 x 10 <sup>4</sup>
No 3 - Suc / SYTO62	40.3591	2.0 x 10 <sup>5</sup>	

Table 24. Comparison of viable count and CellFacts II count obtained from extracted samples prepared with either nycodenz (Nyc) or sucrose (Suc).

The results above confirm that sucrose (60% (w/v)) is a valid alternative to nycodenz.

3.6.3.2 Evaluation of Results

86 samples were analysed of which 15 were culture positive i.e.  $> 10^3$  cfu ml<sup>-1</sup> as detected by either plate culture or Easicult (dipslide). This is a very small database, however, each sample was analysed twice, once with each of the two dyes.

The instrument data were compared against the collated culture data. The resulting sample analysis breakdown is as shown in Table 25.

True +ve	True -ve	False +ve	False-ve
15	45	26	0

Table 25. Breakdown of sample analysis by comparison of CellFacts II data with microbiological culture data, taking the latter as the reference. Data returned against analytical rule 1 (Table 26).

No false negatives were reported. Although there were 15 culture positive samples, the instrument reported a further 26 samples as being positive i.e. potentially 26 false positives. These data were returned using “rule 1” set out in Table 26.

Rule No.	Result	Rule Definition (CellFacts II Data Output)
1	positive	TVC for both DiSC <sub>3</sub> and SYTO 62 $> 1.5 \times 10^4$ and average fluorescence for DiSC <sub>3</sub> $> 40$ and for SYTO 62 $> 30$
2	positive	if both TVCs were above $2.5 \times 10^4$ and the average fluorescence values $> 20$
3	high cell biomass	If SYTO 62 count was $> 1.5 \times 10^4$ and the average fluorescence $> 30$ and DiSC <sub>3</sub> count $< 1.5 \times 10^4$ , then the sample contained a high (dead) cell biomass.
4	sample at risk	TVC values for both DiSC <sub>3</sub> and SYTO 62 were $> 1.0 \times 10^4$ with corresponding high average fluorescence values.
5	negative	Where none of the above applied

Table 26. Analytical rules applied to the CellFacts II data.

Further analysis of the “false +ve” samples revealed that there were two categories of samples not reported by cell culture:

- Samples showing a high cell biomass primarily consisted of dead cells, for example, where a significant microbial population was present after challenge with biocide. This situation includes a significant risk of a percentage of cells remaining viable with the potential to re-establish an unacceptable level of contamination, e.g. recovery of injured cells.
- Samples showing significant TVC values, i.e.  $> 1.5 \times 10^4$  for both DiSC<sub>3</sub> and SYTO 62, and corresponding high average fluorescence values. These samples had a high microbiological content which did not grow in routine microbial culture. This situation would result in a reduction of the efficiency of the biocide opening the possibility of growth of “culturable” microbes and compromise of product due to a (previously) undetected high microbial biomass.

The database showed that the instrument performance was as given in Table 27.

	Application of Rules 1 and 2	Inclusion of "at Risk" Samples with true +ves (Rules 1,2 and 4)	Inclusion of "High Biomass" Samples with true +ves (Rules 1,2,3 and 4)
Sensitivity	100%		
Specificity	63.4%	80.4%	97.8%
Positive predictive	36.6%	57.7%	93.75%
Negative predictive	100%		

Table 27. Performance of CellFacts II.

The above data facilitates risk assessment of the microbiological content of a slurry effectively in real-time. The data also presents the opportunity for real-time evaluation of the effectiveness / efficiency of a biocide and subsequently, biocide dosing to match the microbiological load of the slurry.

It should be noted that comparison of the microbiological culture techniques employed (dipslide



and plate culture) by application of the same performance criteria as applied to CellFacts II shows the inadequacies of the dipslide method. From the database the dipslide sensitivity is ~ 52.4% and the negative predictive 86.7%. Specificity and positive predictive are 100%, however, these values are misleading since they are derived by reference to culture data only (should be compared with an alternative means of quantifying viable bacterial cells e.g. fluorescence microscopy). The rules used above were developed from detailed consideration of the raw and algorithm data generated by CellFacts II.

### 3.7 Conclusions

Clearly then, the microbial diversity in White Pigment Slurries is greater than previously assumed, with a number of common soil and water bacterial species being detected and identified by employing a combination of culture, microscopy and molecular techniques. One suspects that the isolates identified by 16s rRNA sequencing to date comprise only a small proportion of the total range of species present, and it may be that analysis of total population DNA extracts will be required for complete elucidation of the microbial diversity.

It was only possible to detect the presence of SRBs and other anaerobes in the production process of White Pigment Slurries when there were long retention times and the introduction of oxygen was prevented, i.e. in clarifying and settling basins as well as in storage tanks with insufficient mixing. The greying of a slurry as well as a significant increase in the viscosity caused by anaerobic growth is still a major problem within the paper industry where additional preservation as well as the microbiological quality control is still not given the necessary attention.

Information about the physiologic state of a cell is a very important factor with regard to the optimisation of the preservative performance. The fast detection of microbial growth in a slurry by means of measuring methods in real time also enables additional preservation with biocides to be carried out early so that the cells can be attacked during their phase of exponential growth.

By means of a procedure by which cell activity is rapidly assessed and additional preservation

using the appropriate bactericidal quantities of preservative carried out, biocide use can be optimised and adaptation (resistance) of bacteria to preservatives can be minimised.

## **CHAPTER 4**

### **Preservatives / Disinfectants**

#### 4.1 Introduction

Biocides have become indispensable for ensuring the high hygiene and health standard of our modern society. Microorganisms - such as bacteria, mould, algae – and viruses, for example, are omnipresent. They endanger our health and products become unusable due to contamination. Remedial action is primarily taken by using chemical substances with which the harmful organisms can be combated. The definitive action of biocides is to act selectively against harmful organisms.

Preservatives (biocides) are defined chemical substances or mixtures of substances having a low toxicity and good skin tolerance. Low concentrations of them, usually in the range of 0.1 - 5000  $\mu\text{g ml}^{-1}$  or g, destroy microorganisms or inhibit their development while showing good compatibility with the product they are used to protect.

The products in use today are mature and reliable with respect to their effectiveness. New active agents are rarely found these days, apart from modifications of known substances due to strong restrictions and limitations through legislation, health and safety.

Within Omya, other pigment industries as well as the paper and paint industries biocides were being used but there were only a few evaluations which took into account the microbiology. However, in the course of this thesis it was possible to develop and even patent new active agents for the preservation of White Pigment Slurries. Furthermore, it was possible to understand the mechanism of action of existing biocides and to adapt them to the respective application.

#### 4.1.1 Bacteriostatic and Bactericidal Effects

Antibacterial action may be either bacteriostatic or bactericidal. Bacteriostasis is the term used to describe the prevention or inhibition of growth by an agent when measured under conditions where growth would normally occur. The effect is reversible, such that, if the agent is removed or neutralised, the cells will recommence growth and cell division. Bactericidal effects occur when bacterial cells exposed to an agent are not recoverable (i.e. do not recommence growth) after removal or neutralisation of the agent. This is due to an irreversible lethal process taking place in the cell.

Under laboratory conditions, where the behaviour of populations rather than individual cells is investigated, differences between bacteriostatic and bactericidal effects may be difficult to distinguish in a situation where some cells may be dividing and others dying, i.e. a dynamic situation.

Mechanism of action studies indicate that some antimicrobial agents are primarily bactericidal whilst others are mainly bacteriostatic. Some agents may also exert bacteriostatic or bactericidal effects depending on their concentration. In studying mechanisms of action it is important to consider that although cell damage may result in bacteriostasis, it is the inability of the cells to make good this damage which produces a bactericidal effect. Thus, for example, with membrane-active antibacterial agents such as phenols and chlorhexidine, although loss of metabolites through the damaged membrane inhibits cell growth, it is the extent of the membrane damage and the inability of the cells to make good this loss on neutralisation of the agent, which ultimately produces bactericidal effects.

#### 4.1.2 Mechanisms of Antibacterial Action

There is a growing awareness of the need to establish mechanisms of action for antibacterial agents. This information assists in the design of new compounds or combinations of compounds and in the understanding of resistance mechanisms, and provides a focus for toxicological attention. Certainly, mechanism of action studies have shown that biocidal agents (preservatives, disinfectants) can no longer be considered under a crude umbrella as general cell poisons;

furthermore their activity may be optimised by design.

Target regions for antibacterial agents can be classified very conveniently as the cell envelope, cytoplasmic membrane and cytoplasm. Within these broad areas of the cell a further division of targets can be made (Figure 43). It should be remembered that these divisions are accepted for convenience only and do not represent mutually exclusive areas for biocide interaction. Indeed, many of the antibacterial agents currently in use will have more than one target within the bacterial cell, and it is axiomatic that the vital interdependence of cellular functions must not be ignored.

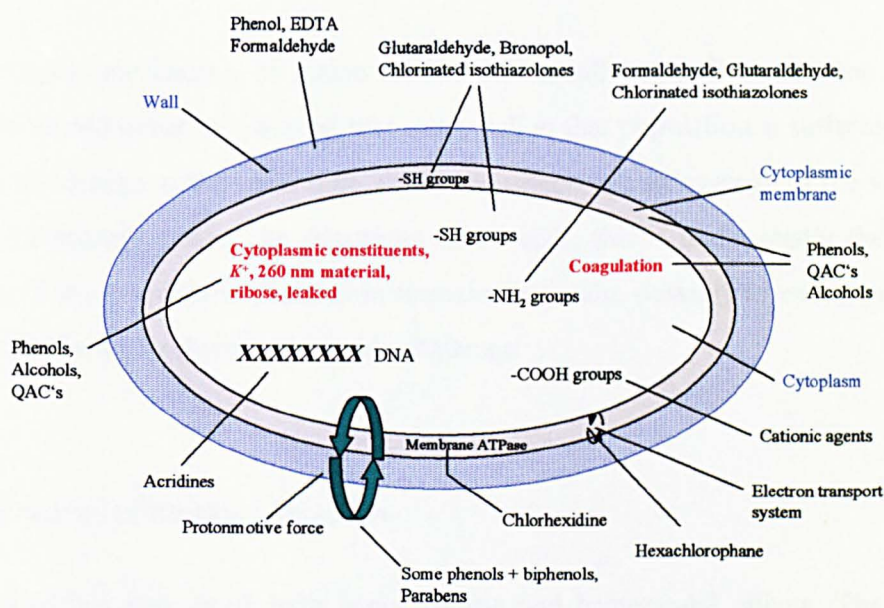


Figure 43. Mechanisms of antibacterial action (after Denyer & Hugo, 1991).



#### 4.1.3 Variation in Mechanism of Action

It is important to remember that the activity of compounds may well result from effects on several cell functions and the relative importance of each effect may vary depending upon the value of that function to the species of organism challenged. Furthermore, an altered sensitivity towards antibacterial agents may reflect changes in the accessibility of targets within the cell and again may alter significantly the principal lesion(s) responsible for antibacterial activity. Such alterations in sensitivity may arise through changes in the physiological state of the organism dependent upon growth phase, environment and selective pressures.

Most importantly, mechanism of action studies can usually only determine the bulk population behaviour. It should never be assumed that every cell in that population is suffering the same type, and degree of, damage at the same time. Observed effects are an average of the whole population and do not necessarily reflect the sensitivity of all cells; this is undoubtedly the basis by which surviving fractions in a treated population sometimes remain, possibly to emerge more adequately equipped to deal with a subsequent biocide challenge.

#### 4.1.4 Consequences of Biocide Interaction

Antibacterial agents may exert both bacteriostatic and bactericidal effects. The mechanisms of action responsible for each effect may not necessarily be the same. Bacteriostatic effects can be considered generally to represent some form of metabolic inhibition which is released upon removal of the biocide, while bactericidal action is caused by irreversible or irreparable damage to a vital structure or function of the cell. Quite possibly, damage arising from interactions with biocides may, in many instances, be repairable but, because of inhibition at another metabolic site, appropriate repair processes cannot be initiated and cell death occurs.

#### 4.1.5 Factors Affecting Antibacterial Activity

Accurate interpretation of the results of action studies depend on a clear understanding of the factors which may affect the activity of an agent in relation to biochemical effects. In many cases these factors are deliberately exploited for elucidating mechanisms of action. The activity of antimicrobial agents against micro-organisms depends on two major factors – the nature of the physical environment and the condition of the organism.

Pretreatment factors:               - conditions used for cultivation of bacterial cells

Factors during treatment:       - concentration of antibacterial agent  
  - temperature  
  - number of organisms  
  - environmental pH  
  - constituents of the suspending medium  
  - type of organism

#### 4.2 Regulatory, Safety and Environmental Issues

Since mineral dispersions might be used in the production of food packaging the substances used have usually to comply with most of the relevant laws, such as BgVV Germany, FDA USA and current and emerging EU directives.

With respect to the relevant laws in different countries not only the quality of the biocides but also possible limitations of the quantity have to be considered. Special attention has to be paid to the fact that different countries may also impose different limitations on the quantity. Below, we identify some of the most relevant criteria applicable today.

#### 4.2.1 Biocidal Products Directive 98/8/EC (BPD)

On the 24<sup>th</sup> April 1998, Directive 98/8/EC (Biocidal Products Directive) was published in the Official Journal of the European Communities, Vol. 123 and implementation of the Directive must have taken place in all member states by 14<sup>th</sup> May 2000.

The purpose of the Directive is to control the placing on the market of biocidal products by introducing a scheme for mutual authorisation (provided that active ingredients are listed in Annex I, IA or IB of the Directive) and includes all non-agricultural pesticide applications.

Some non-agricultural pesticide regulations already exist in a number of member states including Belgium, Denmark, Ireland, Finland, Netherlands, Sweden and UK. Essentially, the BPD will extend and standardise these regulations across the European Union whilst also introducing a positive list of active ingredients.

Data requirements (specified in Annexes II, III and IV to the Directive) are extensive and include information not only on toxicity and ecotoxicity but also application details, active ingredient and product identity, physical and chemical properties, methods of detection and identification, efficacy and use, risk management measures and classification and labelling.

The cost of generating and providing the above data is high and could be as much as EURO 3'000'000 for an active substance's inclusion in Annex I and EURO 150'000 for authorisation of a biocidal formulation.

The Directive stipulates a 10-year period of data review due to the complexity of the work involved.

Whatever the final outcome, it is clear that this complicated directive will significantly affect a wide variety of biocide consumers in Europe that have been otherwise largely unregulated.

#### 4.2.2 Risk for Humans and Environment

The requirements with respect to the toxicity of an antimicrobial substance primarily depends on the intended use. The most rigid requirements have to be met by products used for food preservation as people are exposed to high concentrations (0.05 – 0.50%) over a long period of time, often daily. For this reason it is most important that these substances are recognised as safe for health.

With respect to the intended use it is necessary to consider not only the quality of the biocide but also possible limitations of the quantity especially with respect to biodegradability, air pollution, by formaldehyde for example, and the risk of skin-sensitisation for humans. To review this area of legislation is beyond the scope of this thesis.

#### 4.3 Constraints on Suitable Types of Preservatives / Disinfectants

Today, biocides for the preservation of mineral slurries have to meet far more requirements than showing demonstrable bactericidal action alone. Some of the most important criteria which decide whether a bactericidal agent can be used for the preservation of mineral slurries can be summarised as follows:

- Has to be thermally stable up to a minimum of 60°C.
- Needs to have a positive redox potential.
- Its inhibition of nitrification has to be < 30% (in dilution) in slurry so as not to impair the nitrification in subsequent waste water purification plants.
- It has to be biodegradable to a level greater than 80% (w/w), preferably 100% (w/w) (OECD 301D).
- May not generate a negative inhibition area around finished paper or application surfaces - this applies to the need to prevent extraction of biocide from paper and packaging materials when in contact with foodstuff.
- Must have no negative influence on other mineral dispersion properties.
- Required to have Regulatory Approval (e.g. FDA, BgVV, Nordic Ecolabelling).

Among a large number of bactericidal agents the following ones have proved to be successful as preservatives in mineral dispersions. Once again, this list should not be considered as exhaustive: many biocides which have proven efficacy have either fallen out of favour due to regulatory requirements or even environmentally-related perceptions, and many biocides remain to be discovered or, if already existing, to be applied in this field. We go on to consider some currently used biocides and identify a newly applied biocide in this field.

#### 4.4 Preservatives / Disinfectants in the Pigment and Paper Industry - Experimental Investigations

Minimal inhibitory concentration (MIC) figures published in the literature have been generated for use in water (Wallhäuser, 1995). These, however, are not appropriate for use in White Pigment Slurries and are of little value. As a consequence all MIC studies for the application of White Pigment Slurries have been redone. All analyses (MIC / HPLC) have been repeated three times with high reproducibility.

##### 4.4.1 2-Bromo-2-Nitropropane-1,3-Diol (Bronopol)

Bronopol has a broad spectrum of antibacterial activity and belongs to the group of aldehyde-releasers as well as to the group of activated halogen-compounds. It is widely used as a preservative in pharmaceutical and cosmetic products (Croshaw *et al.*, 1964; Storrs & Bell, 1983). Another important emerging application is the use as a preservative in the filler and pigment industries (e.g. calcium carbonate slurries).

There have been a number of studies of the mechanism of action of bronopol, all of which conclude that activity relates to interaction with essential thiols within the cell (Stretton & Manson, 1973; Bryce *et al.*, 1978; Wong & Preece, 1985). Such interaction is thought to lead to oxidation of thiols through a radical anion intermediate. Earlier studies on the mode of action have shown that in aqueous solution cysteine is rapidly oxidised to cystine in the presence of bronopol (Stretton & Manson, 1973). The bronopol also acts as an oxidative catalyst in the presence of

oxygen. In the absence of air the reaction is much slower and the bronopol disappears at a comparable rate to the cysteine (Shepherd *et al.*, 1988).

Such chemical information is compatible with the observed biological effects of bronopol. Following the addition of bronopol to actively growing cultures of bacteria, growth ceases immediately for a period dependant upon the concentration applied. After this induced bacteriostasis, growth proceeds at an inhibited rate.

The minimum growth inhibitory concentration (MIC) is 10 - 15 ppm bronopol. For pigment slurry preservation 35 ppm bronopol are recommended (Table 28).

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	5 ppm <sup>1)</sup>	15 ppm <sup>1)</sup>	25 ppm <sup>1)</sup>	35 ppm <sup>1)</sup>	50 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	1.4 x 10 <sup>5</sup>	100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	-	4.6 x 10 <sup>5</sup>	< 100	< 100

<sup>1)</sup> The concentrations of bronopol were calculated as active on dry metric ton (dmt) slurry.

Table 28. Minimum growth inhibitory concentration of bronopol.

Bactericidal activity, but not growth inhibition, may be significantly reduced by the exclusion of air or inclusion of the enzymes catalase or superoxide dismutase in cell suspensions.

It is apparent that bronopol undergoes two distinct types of reaction with accessible thiols within cells. Under aerobic conditions, bronopol catalyses the oxidation of thiols such as glutathione and cysteine to their disulphides, utilising atmospheric oxygen as the oxidant and generating an anoxic state. Further oxygen diffusing into the system will be used catalytically in this manner so long as bronopol remains. Such reactions will bring about the immediate cessation of bacterial growth. By-products or intermediates in this aerobic reaction are active oxygen species such as superoxide and peroxide. These are directly responsible for the bacterial activity of the compound and for the reduced rate of growth following the bacteriostatic period. Once an anoxic state has been created



in the reaction mixture it will be maintained by the presence of unreacted bronopol and reduced thiols but it will allow the slower, consumptive reactions of bronopol to predominate until all the bronopol is consumed. Only then may enzymes such as glutathione reductase restore the redox state of the cell and allow growth to resume. Whether this occurs depends upon the extent of free radical damage incurred during the interim period.

By using bronopol the content of volatile formaldehyde can be significantly reduced. The released formaldehyde can be calculated from the degradation pathway given below:

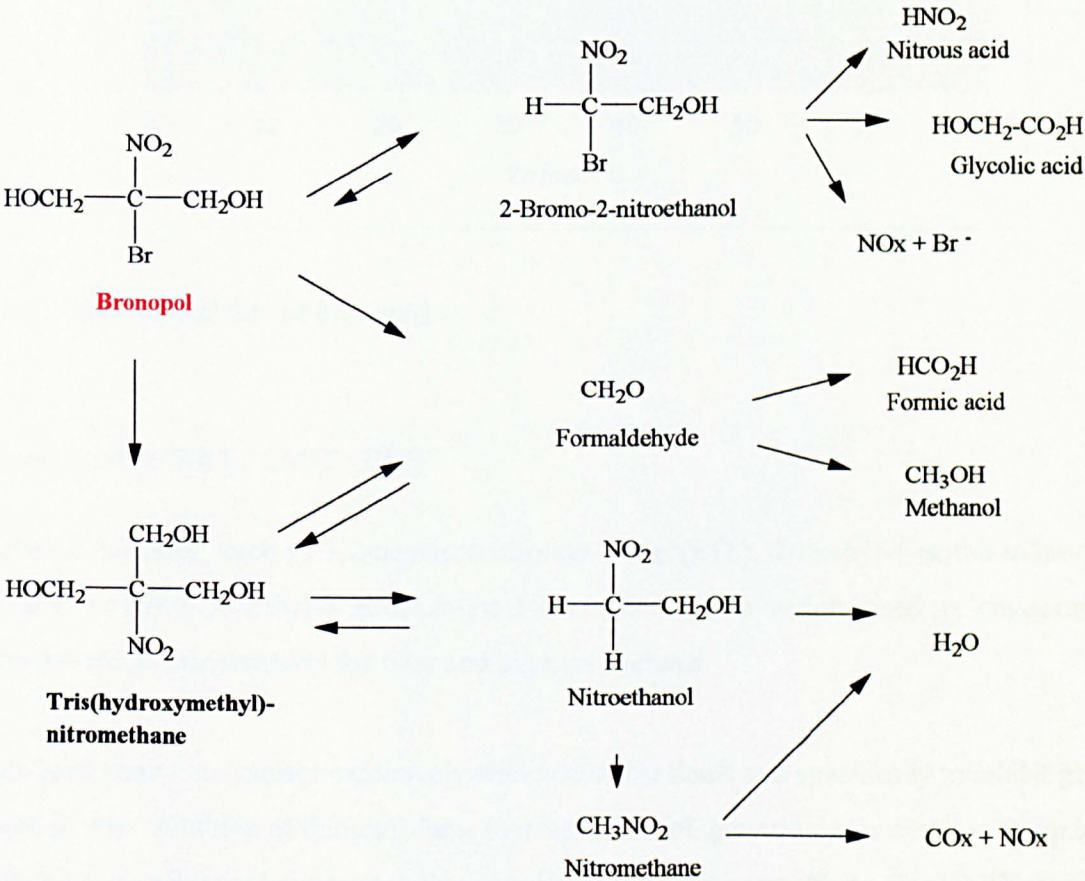


Figure 44. Degradation pathway of bronopol. (Modified from Bronopol Product Information - Knoll MicroCheck, 1997)

A considerable disadvantage, however, is that the compound is not heat-stable. At a temperature of 60°C under alkaline conditions the active agent is completely decomposed within a few hours (Figure 45).

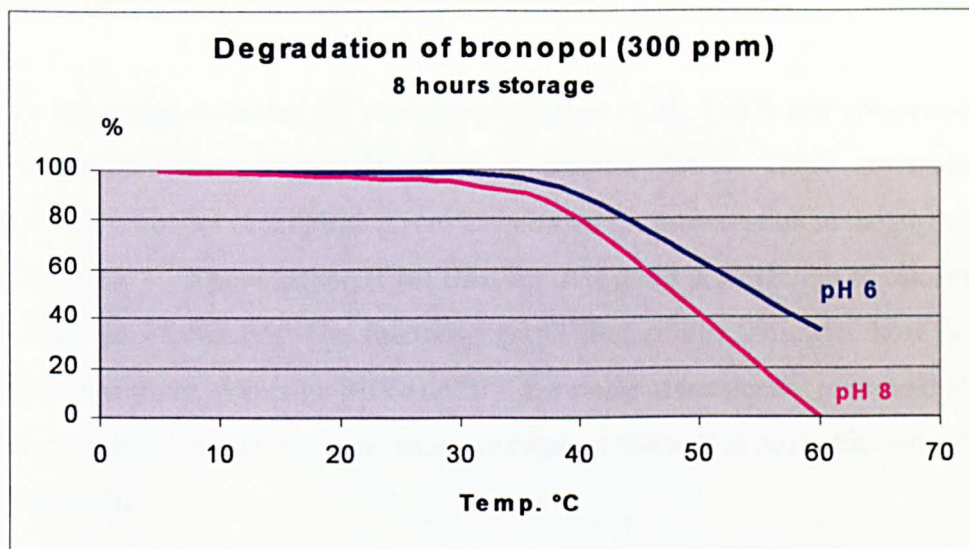


Figure 45. Thermal stability of bronopol.

#### 4.4.2 Isothiazoline (MIT / CMIT / BIT)

Isothiazoline biocides, such as 1,2-benzisothiazoline-3-one (BIT), 2-methyl-4-isothiazoline-3-one (MIT) and 5-chloro-2-methyl-4-isothiazoline-3-one (CMIT) are widely used as environmental biocides as well as preservatives for filler and pigment systems.

BIT has been shown to interact oxidatively with accessible thiols and specifically to inhibit glucose transport *in vivo*. Addition of thioglycollate, mercaptoethanol, glutathione or cysteine at equimolar concentrations to all three biocides totally neutralises their inhibitory effects. For CMIT, activity is also reversed *in vivo* by the addition of valine or histidine.

Initial reaction leads to the formation of a disulphide conjugate. Reaction of the conjugate with further thiols leads to the formation of a thiol dimer and a ring-opened form of the biocide which itself could serve as a further source of interactive thiol to give a dimerised biocide as an additional



product. CMIT and MIT are thought to act similarly to BIT but whilst BIT is a hypersensitising agent, CMIT is both a primary skin irritant ( $> 25 \mu\text{g ml}^{-1}$ ) and Ames test positive. CMIT has reported bactericidal activity, unlike BIT which is primarily bacteriostatic in action. CMIT is antibacterial at considerably lower concentrations than BIT.

Isothiazoline has a high potential for sensitising (Weaver *et al.*, 1985) and compounds based on isothiazoline are not heat-stable (Willingham & Mattox, 1990). These are mainly used in combination with bronopol or ethylene glycol hemiformal for preservation in the pigment and filler industries. By using 3 - 5 ppm kathon (CMIT/MIT = 3:1) good preservation of calcium carbonate slurries is achieved (Table 29). The following graph (Figure 46) illustrates how fast CMIT is degraded by temperature. Although MIT and BIT are stable at temperatures around  $60^{\circ}\text{C}$  -  $80^{\circ}\text{C}$ , preservation with MIT or BIT alone (even a combination thereof) is not sufficiently effective and thus not meaningful.

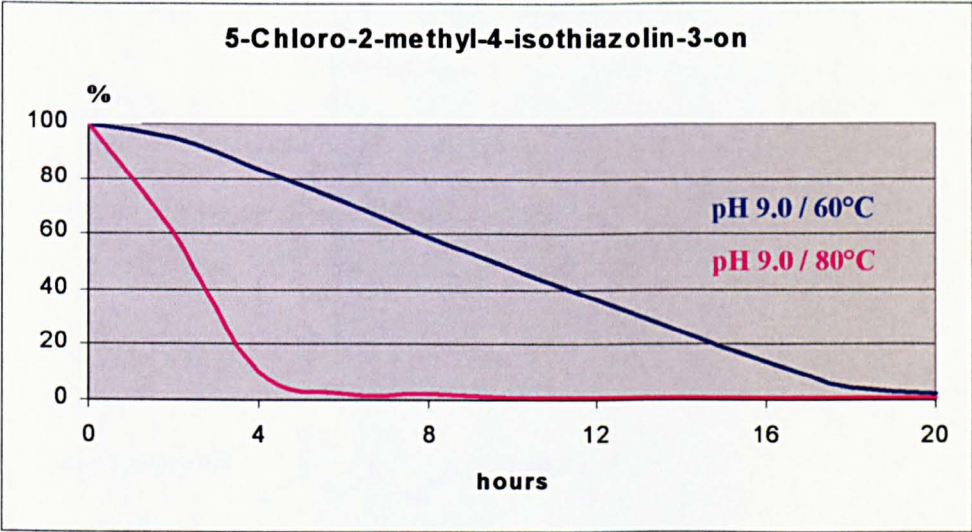


Figure 46. Thermal stability of 5-chloro-2-methyl-4-isothiazoline-3-one (CMIT). The analysis were repeated 3 times with a high reproducibility.

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	1 ppm <sup>1)</sup>	3 ppm <sup>1)</sup>	5 ppm <sup>1)</sup>	10 ppm <sup>1)</sup>	20 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	< 100	< 100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	200	< 100	< 100	< 100

<sup>1)</sup> The concentrations of MIT/CMIT were calculated as active on dmt slurry.

Table 29. Minimum growth inhibitory concentration of MIT/CMIT

Isothiazolones degrade biologically, physically and chemically to form a series of products (Figure 47):

### Degradation Pathway of MIT and CIT

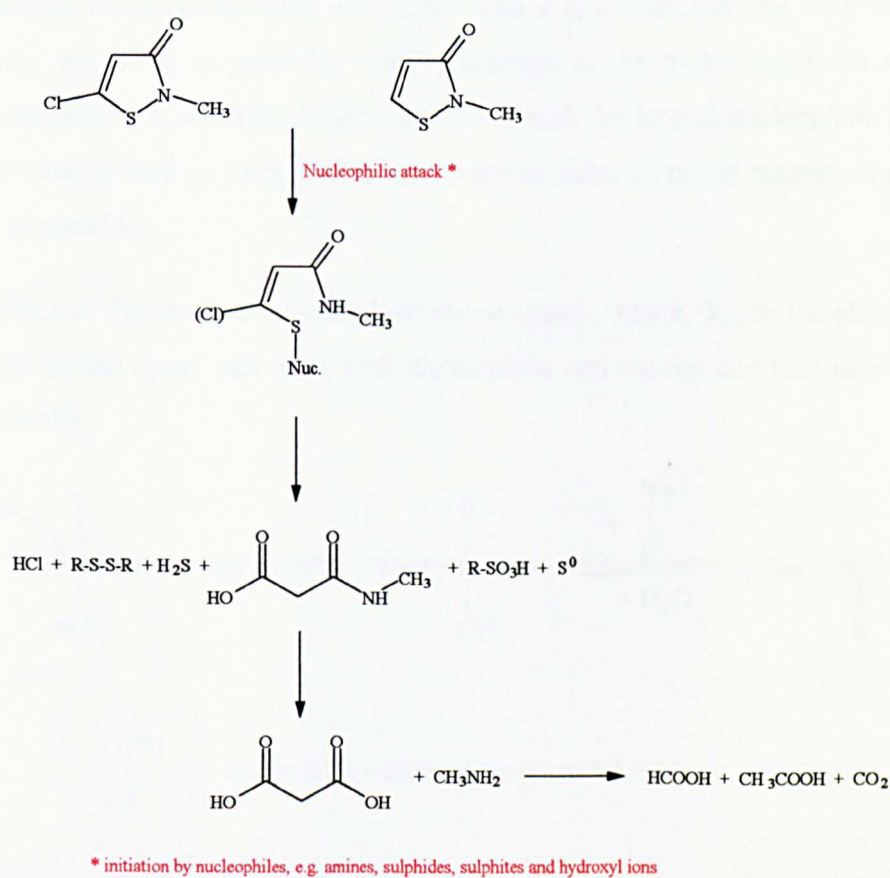


Figure 47. Degradation pathway of MIT and CMIT. (Modified from BODE Chemie, 2000).

#### 4.4.3 Aldehydes (e.g. glutaraldehyde)

Aldehydes have a broad spectrum of activity. Bacteria, fungi, spores and viruses are killed or inactivated. This, however, depends on the effective exposure times.

The pressure from the pigment and filler industries to abstain from using formaldehydes is increasing. Considering that 200 ppm per tonne pigment/filler have to be used for a sufficient preservative action, it is clear that in the production of paper (wet end), quantities of formaldehyde can be released. Due to the high vapour pressure of formaldehyde, vapour is frequently released into the air during and after the disinfection or preservation procedure and concentration levels can build in enclosed production environments. Furthermore, this active agent has been classified as sensitising.

The family of aldehydes can undergo a variety of reactions with one or even two co-reactants of different structure. Such co-reactants are atoms with a lone electron pair with their associated electron density not being required by other groupings in the molecule. If the aldehyde finds different co-reactants in a substrate it favours the one with the largest nucleophilic potential, with which the most stable final product is obtained or the equilibrium of the reaction is as close to the final product as possible.

Aldehydes belong to the group of electrophilic active agents, which, due to the electron deficiency at the carbonyl carbon atom, can react with nucleophilic cell entities and thus exert antimicrobial activity (Figure 48).

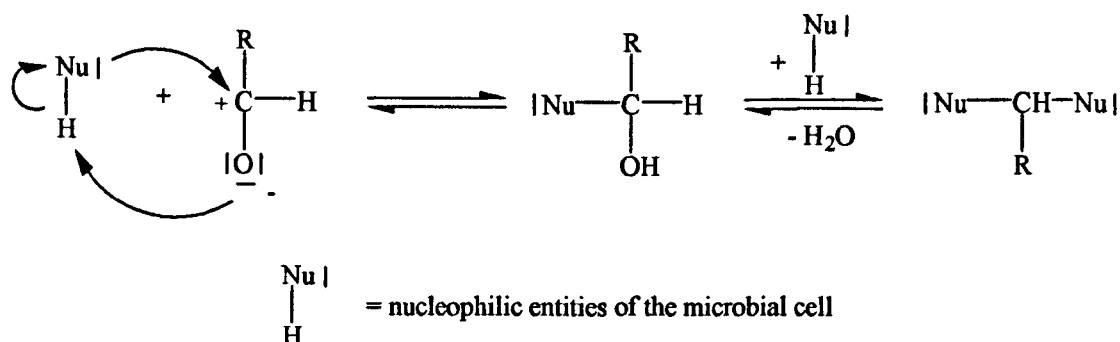


Figure 48. Reaction of aldehyde with nucleophilic cell entities. (Modified from BODE Chemie, 2000).

Microorganisms offer a series of basically suitable reactants such as, for example, aliphatic hydroxyl groups of sugars and amino acids (serine, threonine), primary amino groups in the side-chain residue of peptides as well as acid amide groups. A reaction with aliphatic hydroxyl groups is rather unlikely to occur. It is particularly the comparatively low stability of the aldehyde-alcohol addition compound that makes these compounds suitable active agents in disinfectants.

Aldehydes react with the terminal amino group in the lysine side-chain residue. The inactivation of coliphages, for example, is accelerated by formaldehyde at pH values above 7.8. This leads to the conclusion that the formaldehyde has a functional group as a co-reactant which, from a pH value of 7.8, is in a neutral, i.e. reactive state. This is exactly the case with the amino groups in the lysine side-chain residue of proteins (Wallhäusser, 1995).

#### 4.4.4 Formaldehyde-Releasing Compounds (e.g. Ethylene Glycol-Hemiformals)

Formaldehyde, as such, is often too volatile and too reactive to be used as a microbiocide for the protection of white minerals. It additionally produces unwelcome side-effects such as an increase in viscosity, and has an insufficiently balanced range of activity. One, therefore, continually searches for formaldehyde-releasing compounds which do not exhibit the disadvantageous formaldehyde effects but maintain or even improve the antimicrobial action of formaldehyde. However, the environmental limitations on released formaldehyde still apply.

Formaldehyde-releasing compounds can be found as both solids and liquids, water soluble or oil soluble, alkaline, neutral, or slightly acidic. According to their composition, the ethylene glycol-hemiformals are especially effective against bacteria and therefore useful for the so-called "in-can", or storage container, protection of a large variety of industrial fluids, mainly together with other active ingredients, e.g. fungicides. Also, the protection of white mineral slurries using ethylene glycol-hemiformal has proven to be effective.

Formaldehyde releasing compounds can be solids or liquids, water soluble or oil soluble, alkaline, neutral, or slightly acidic.



The reaction of formaldehyde with alcohols (R-OH), easily takes place under neutral or weakly alkaline conditions and leads to the formation of hemiformals which are relatively heat resistant and remain in equilibrium with the starting products. However, the equilibrium is widely shifted to the side of the hemiformal; free formaldehyde is detectable in mere traces. Under acidic conditions the reaction goes on to produce formals.

The only formaldehyde releasing biocide in use in the white pigment industry is ethyleneglycol-hemiformal.

The addition of formaldehyde to ethylene glycol leads to mono- and bishemiformal of ethylene glycol with different microbicidal efficacy (bishemiformal>monohemiformal).

It has to be mentioned that the dihemiformal has no microbicidal efficacy (Figure 49).

According to their composition the ethylene glycol-hemiformals are especially effective against bacteria and therefore useful for the in-can protection of a large variety of industrial fluids, mainly together with other active ingredients, e.g. fungicides. Also for the protection of white mineral slurries the ethylene glycol-hemiformal in concentrations of 200-250 ppm is useful (Table 30).

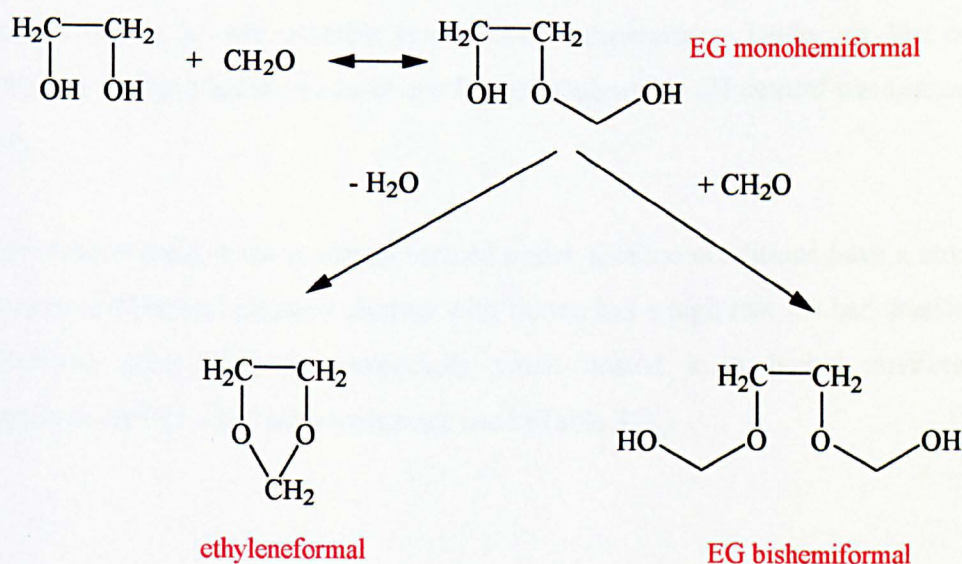


Figure 49. Addition of formaldehyde to ethylene glycol (EG) leads to mono- and bishemiformal of EG. (Modified from BODE Chemie, 2000).

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	50 ppm <sup>1)</sup>	100 ppm <sup>1)</sup>	150 ppm <sup>1)</sup>	200 ppm <sup>1)</sup>	250 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	4.0 x 10 <sup>3</sup>	< 100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	> 10 <sup>6</sup>	1.5 x 10 <sup>4</sup>	< 100	< 100

<sup>1)</sup> The concentrations of ethylene glycol-hemiformal were calculated as active on dmt slurry.

Table 30. Minimum growth inhibitory concentration of ethylene glycol-hemiformal.

#### 4.4.5 3,5-Dimethyl-Tetrahydro-1,3,5-2H-Thiadiazine-2-Thione (DAZOMET)

3,5-Dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione has a broad spectrum of high activity which covers bacteria, fungi and yeast, indicating that the substance is a specialised formaldehyde-releasing compound.

The spectrum of activity is attractive for application in a number of industrial systems, e.g. as a broad spectrum microbiocide which prevents fungal blooms in metal working fluid systems. However, there are limitations, including the finding that 3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione is very unstable at increased temperatures. Under alkaline conditions it is found that more degradation products are formed than under pH neutral conditions (e.g. wet-end systems).

The intermediate breakdown products formed under alkaline conditions have a strong smell. The preservation of filler and pigment slurries with thione has a high risk for bad smelling slurries and end products, such as paper, especially when heated in a humid environment. Today, concentrations of 120 - 170 ppm thione are used (Table 31).



HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	60 ppm <sup>1)</sup>	90 ppm <sup>1)</sup>	120 ppm <sup>1)</sup>	150 ppm <sup>1)</sup>	180 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	600	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	7.0 x 10 <sup>5</sup>	< 100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	-	200	< 100	< 100

<sup>1)</sup> The concentrations of DAZOMET were calculated as active on dmt slurry.

Table 31. Minimum growth inhibitory concentration of DAZOMET.

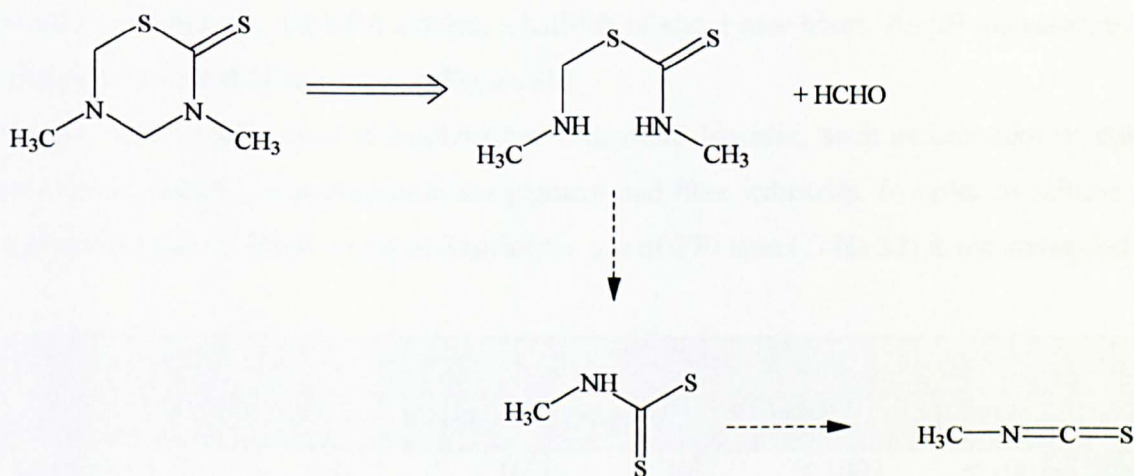


Figure 50. Degradation pathway of 3,5-dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione. DAZOMET hydrolyses in water to formaldehyde and N-methyl-dithiocarbamate ester, which degrades to methylene isothiocyanate (Kvist, 1991).

In 1995, for the first time, thione and its decomposition product came under strong pressure due to a cigarette recall by Philip Morris in America. Cigarettes to the value of more than 200 million US \$ were recalled from the market since methylisothiocyanate and other decomposition products had been found in cigarettes. Later, it was shown that the residual chemical was more like to come from the tobacco plants themselves because, at that time, thione was used as a fungicide as well as being used to prevent rotting during storage. Nevertheless, most board producers in Europe immediately advised us that we should no longer use thione or its decomposition products as preservatives for our slurries (Buri, 1998).

4.4.6 2,2-Dibromo-3-Nitrilopropionamide (DBNPA)

DBNPA has a broad spectrum of high activity with a low environmental impact which covers bacteria, fungi and yeast. It is especially effective against slime forming bacteria (Kato & Fukumura, 1962).

These antimicrobials are fast acting, but are temperature sensitive and will decompose exothermally (liberating heat) at elevated temperatures. DBNPA typically yields a 99.999% microbial kill before it degrades sufficiently to lose effectiveness. At neutral pH and normal system operating temperatures, DBNPA exhibits a half-life of about nine hours. As pH increases, the rate of degradation of DBNPA increases (Figure 51).

They are today mainly used in combination with other biocides, such as bronopol or ethylene glycol hemiformal for preservation in the pigment and filler industries. In order to achieve good preservative action in White Pigment Slurries the use of 270 ppm (Table 32) is recommended.

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	20 ppm <sup>1)</sup>	60 ppm <sup>1)</sup>	120 ppm <sup>1)</sup>	200 ppm <sup>1)</sup>	270 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	-	5.0 x 10 <sup>5</sup>	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	-	-	2.9 x 10 <sup>5</sup>	< 100

<sup>1)</sup> The concentrations of DBNPA were calculated as active on dmt slurry

Table 32. Minimum growth inhibitory concentration of DBNPA.

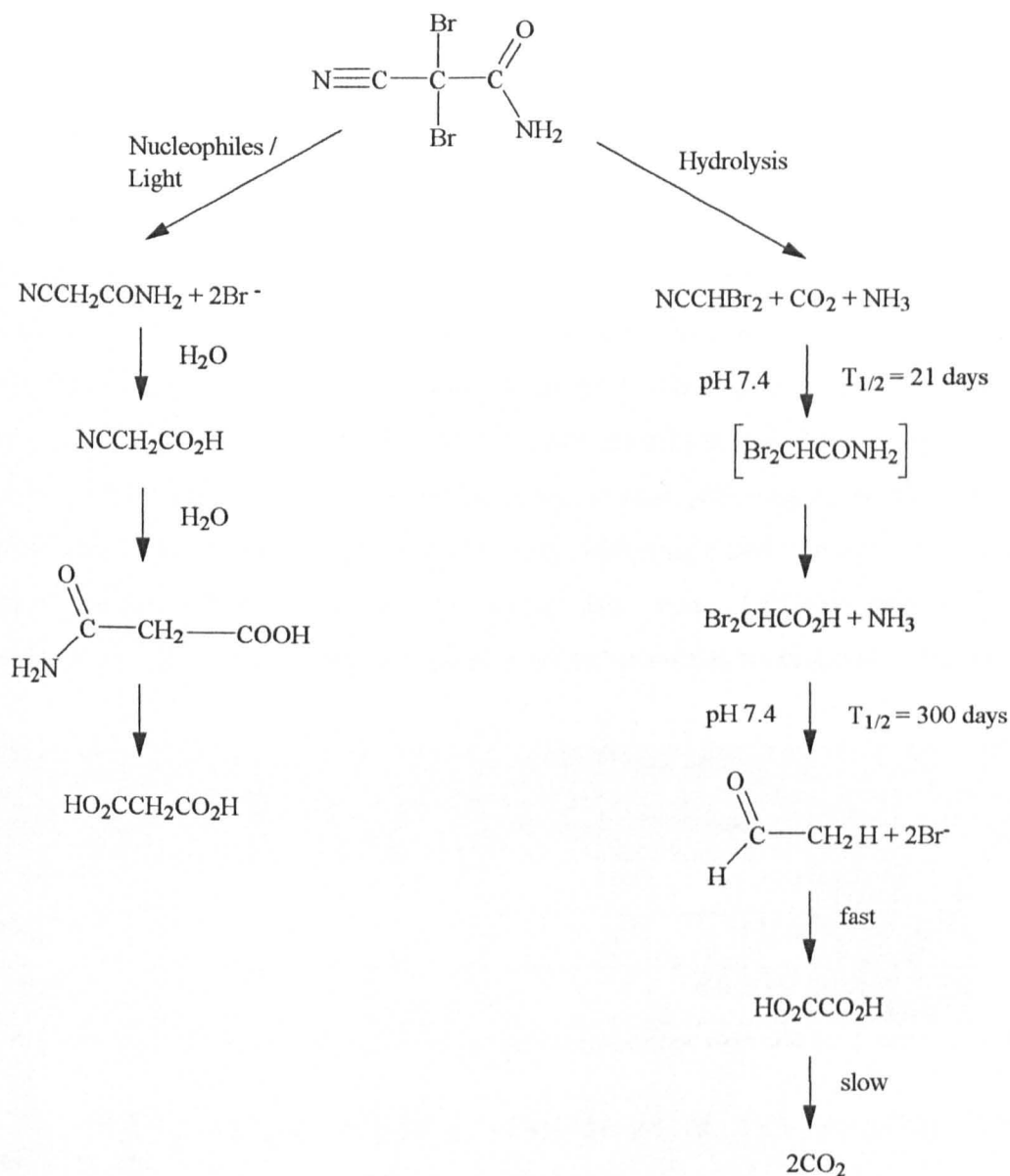


Figure 51. Degradation pathways of DBNPA. (Modified from DOW - Decomposition Pathways of DOW DBNPA, 2000).



4.4.7 1-(3-Chloroallyl)-3,5,7-Triaza-1-Azoniaadamantane Chloride

The efficacy of 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane chloride covers a broad spectrum of bacteria and mould producing fungi, yeast included. However, the activity against bacteria is more pronounced than that against fungi, characterising the microbiocide as a formaldehyde releasing compound.

As a hexaminium salt the active ingredient has a weak cationic character. Nevertheless, it is compatible with anionic White Pigment Slurries. The formaldehyde which is responsible for the antimicrobial efficacy is released independent of the pH of the substrate. The application should, however, be restricted to formulations which do not contain protein (e.g. casein) and to those which tolerate the tendency of the hexaminium salts to cause yellowing especially in formulations containing traces of monomers which may react with the amine compounds released from hexaminium salts (Paulus, 1993). By using 200 ppm 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane good preservation of calcium carbonate slurries is achieved (Table 33).

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	50 ppm <sup>1)</sup>	100 ppm <sup>1)</sup>	150 ppm <sup>1)</sup>	200 ppm <sup>1)</sup>	250 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	> 10 <sup>6</sup>	< 100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	-	4.0 x 10 <sup>4</sup>	< 100	< 100

<sup>1)</sup> The concentrations of 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane were calculated as active on dmt slurry.

Table 33. Minimum growth inhibitory concentration of 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane.



#### 4.4.8 Phenol Derivatives (e.g. o-Phenylphenol)

Phenol, also termed carbolic acid, and the phenol derivatives could also be considered as acids because of their acidity and the resulting capacity to form stable salts. Carbolic acid and soaps have been widely used as cleansers and disinfectants including in the home and in medical environments. Today, a wide range of phenols are used for the protection of materials. Since o-phenylphenol is used to preserve citrus fruits because it has the most favourable toxicity data (Paulus, 1993) this phenol derivative especially has rapidly gained importance as a preservative for the protection of pigment slurries, and this is new.

The free hydroxyl group together with the aromatic system of the phenyl ring constitutes the reactive centre of the phenol molecule. Its reactivity can be influenced in various ways by introducing different substituents into the phenyl nucleus.

Alkylated phenols are less soluble in water and less acidic than phenol. Furthermore, the activity decreases in the direction  $m > p > o$ -substitution. Nevertheless, the alkyl phenols which are of interest as biocides are still capable of forming alkaline salts which dissolve easily in water. With reduced water-solubility the ratio of the distribution between the aqueous and non-aqueous phases including bacterial phases changes, a property which is important for the use of phenol derivatives as microbicides. However, not only the distribution factor changes but also the capability of reducing surface tension changes; in fact it changes increasingly with the length of the alkyl chain. As is to be expected for membrane-active substances, a consequence of these property changes is that increasing antimicrobial effectiveness is observed with increasing alkyl chain length.

As already mentioned, phenol derivatives are membrane-active microbicides. They adsorptively coat the surface of the microbial cell then, at higher concentration, they are dissolved more or less rapidly and/or effectively by lipids, depending on their chemico-physical properties. They attack the cell envelope and penetrate into the cell. Intracellularly, there are reactions within the protoplasm; enzymes are inhibited e.g. the oxidoreductases and the enzymes of carbohydrate and protein metabolism react particularly sensitively. Whether the phenol derivatives act microbistatically or microbicidally is purely a question of the application concentration. At low concentrations in ambient medium, there is only reversible adsorption of the phenolic active

substance at the cytoplasmic membrane and the related inhibiting effect. As stated above the cell envelope is penetrated and destroyed and the microbe killed only at higher concentrations.

For practical use of microbicidal phenol derivatives, it is frequently necessary to improve their solubility, especially their solubility in water, and hence to shift the distribution ratio towards the aqueous phase. If phenol derivatives are converted into sodium or potassium salts, which dissolve easily in water for this purpose, it is important to remember that the dissociated phenolate anion is not nearly as effective as the undissociated phenol. As solubilisation is an urgent requirement for applicability, one has to find a compromise when using alkalis or amines as solubilising agents. Sometimes half of the alkali quantity required for salt formation is sufficient to attain adequate solubility in water; in other cases it is necessary to use excess alkali.

o-Phenylphenol acts according to the scheme which is typical of phenol derivatives. The molecules adsorb on the cell membranes and attack them. The speed of this action is impressive. Pseudomonads are perceived to be in a physiologically stressed state within a few minutes and become non-viable soon afterwards. After the cell membrane has been attacked the cell is permeable to liquids. This was confirmed experimentally. The volume of the cell expanded by approximately 10% within a short time frame (Figure 52). It is, however, not ideal as a disinfectant in slurries due to the relatively low solubility level and its deficiency to be effective against slime-forming bacteria which is discussed in more detail below. Its value in preservation has been established and its application forms are under current patent rights (Schwarzentruher & Buri, 2001. Patent # 01943291.3-2103-EP0104729).

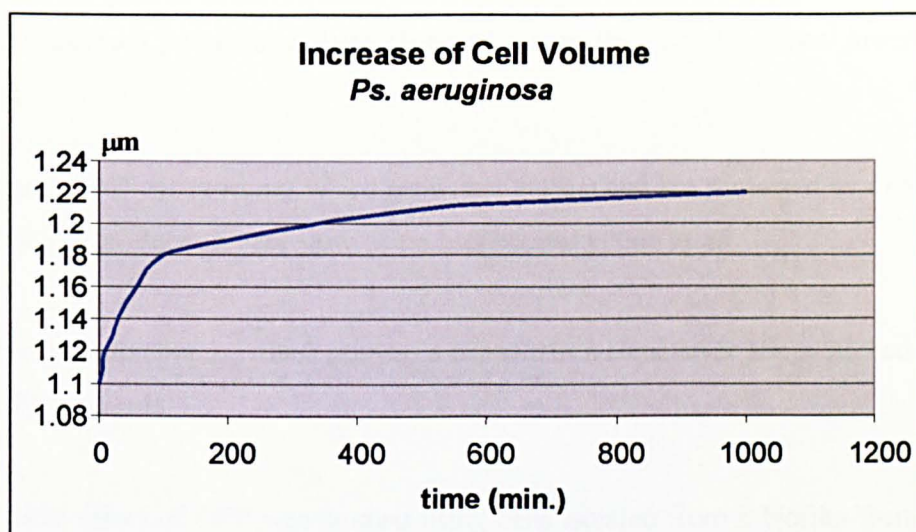


Figure 52. Increase of cell volume of *Ps. aeruginosa* ( $\mu\text{m}$  = equivalent spherical diameter).

Corresponding to the mechanism of action, the microbicidal phenol derivatives are effective against a wide spectrum including bacteria, yeast and fungi. To achieve an optimum preservation of pigment slurries quantities of 200 - 300 ppm o-phenylphenol have to be used (Table 34).

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	100 ppm <sup>1)</sup>	150 ppm <sup>1)</sup>	200 ppm <sup>1)</sup>	250 ppm <sup>1)</sup>	300 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>6</sup>	< 100	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	7.7 x 10 <sup>5</sup>	<100	< 100	< 100
3 <sup>rd</sup> inoculation	> 10 <sup>6</sup>	-	-	100	< 100	< 100

<sup>1)</sup> The concentrations of OPP were calculated as active on dmt slurry.

Table 34. Minimum growth inhibitory concentration of OPP

The action or speed of action, respectively, is significantly reduced if bacteria are protected by a slime layer. Here, two possibilities were observed during the microbiological investigation of the Verpol plant:

- a) In the case of cell communities which grow in a biofilm and are protected as a consequence from OPP i.e. produces a very slow or no bactericidal action at all.
- b) Cells, e.g. pseudomonads, which possess a capsule or a slime layer are protected against attack by an OPP molecule.

The bactericidal effect of OPP was studied using cells isolated from a biofilm from the base of a storage tank. Figure 53 shows that although the cells are subject to physiological stress, which is apparent from the shrinking of the cells, they recover after a short period of time and regain their physiologically active state.

To be able to make use of the positive properties and advantages as well as of the very good preservative properties of OPP it has to be ensured that the substance is added into the system under microbiologically "clean" conditions, i.e., if possible, the TVC in a storage tank or in a rail waggon should be  $< 1000 \text{ cfu ml}^{-1}$ . This can be ensured by taking advantage of the high temperature stability of OPP, i.e. dosing it at temperatures of  $> 60^{\circ}\text{C}$ .

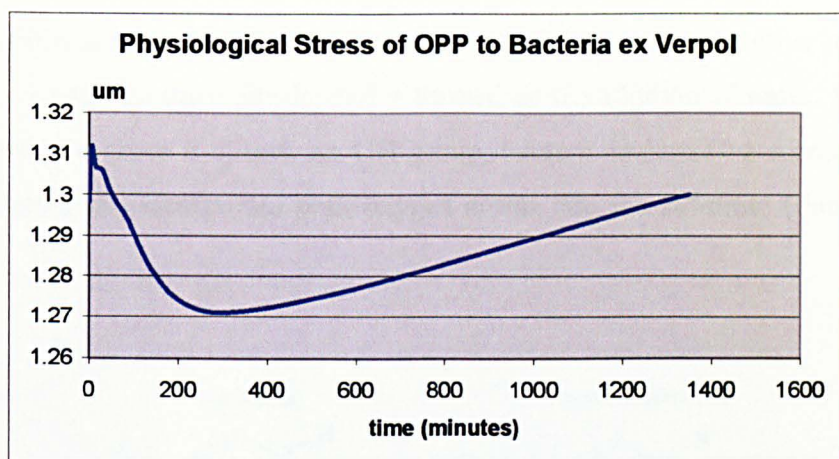


Figure 53. Physiological stress of OPP to slime-forming bacteria ( $\mu\text{m}$  = equivalent spherical diameter).

The precondition for an aerobic degradation of aromatic hydrocarbons such as, for example, o-phenylphenol is that the compounds have a concentration that is beyond the inhibiting peak.

Bacteria, mainly *Pseudomonas*, *Flavobacterium* and *Rhodococcus* species, but also some fungi and yeast like *Aspergillus* and *Candida* species oxidise these aromatics into the key component brencatechine (catechol) as shown in Figure 54.

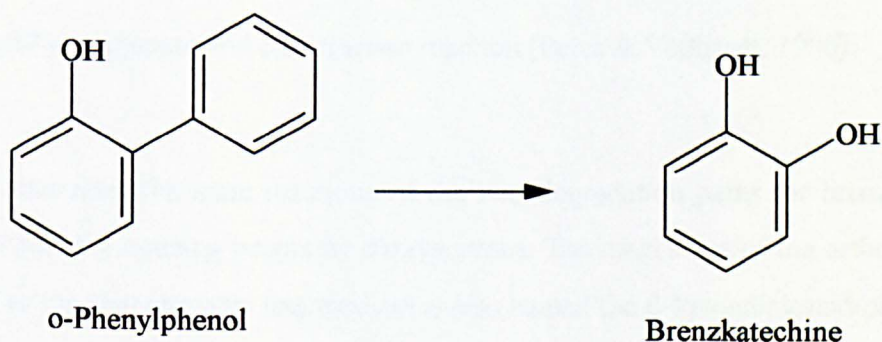


Figure 54. Oxidisation of OPP into brencatechine (Peter & Vollhardt, 1990).

The introduction of two hydroxyl groups in the 1,2- or 1,4-position is the pre-condition for the ring-opening. Two types of enzymes take part in the preliminary oxygenation. The monooxygenases



(A) catalyse the incorporation of an oxygen atom into the substrate, the other enters into H<sub>2</sub>O. In a consecutive reaction a trans-dihydrodiol is formed by the addition of water. If, as in the case of the o-phenylphenol, there is already an OH-group, bacteria undertake a monooxygenase reaction. The dioxygenases (B) incorporate both oxygen atoms into the substrate (double hydroxylation) (Figure 55).

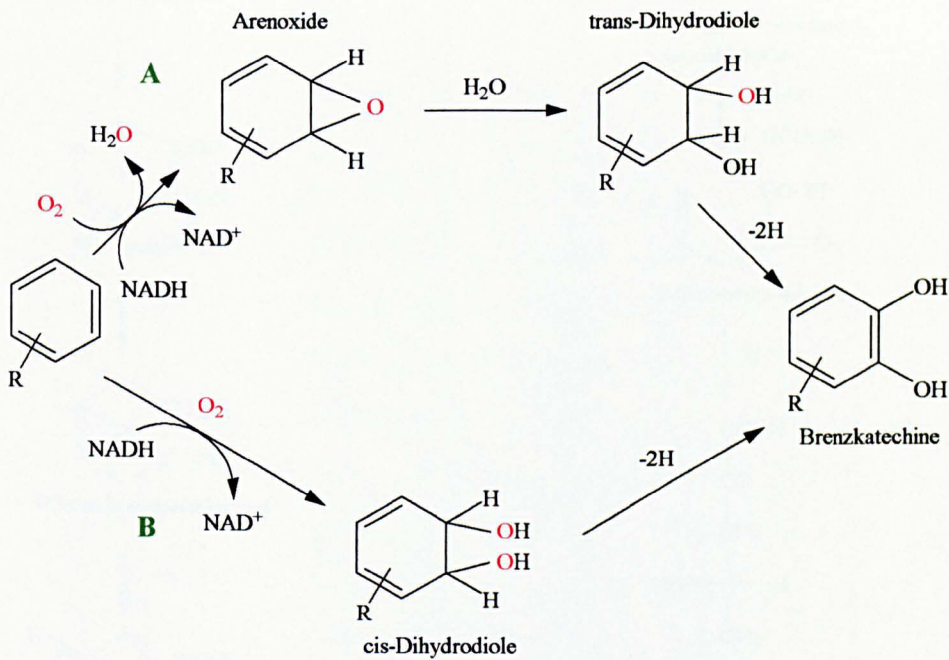


Figure 55. Monooxygenase and dioxygenase reaction (Peter & Vollhardt, 1990).

Figure 56 illustrates the main reactions of the two degradation paths for brenzkatechines. With both paths the ring-opening occurs by dioxygenases. The main steps of the ortho-pathway, which according to the characteristic intermediate is also named the β-ketoadipicacid-pathway, comprise a lactonisation, isomerisation and hydrolysis of the ring-opening product into succinate and acetyl-CoA.

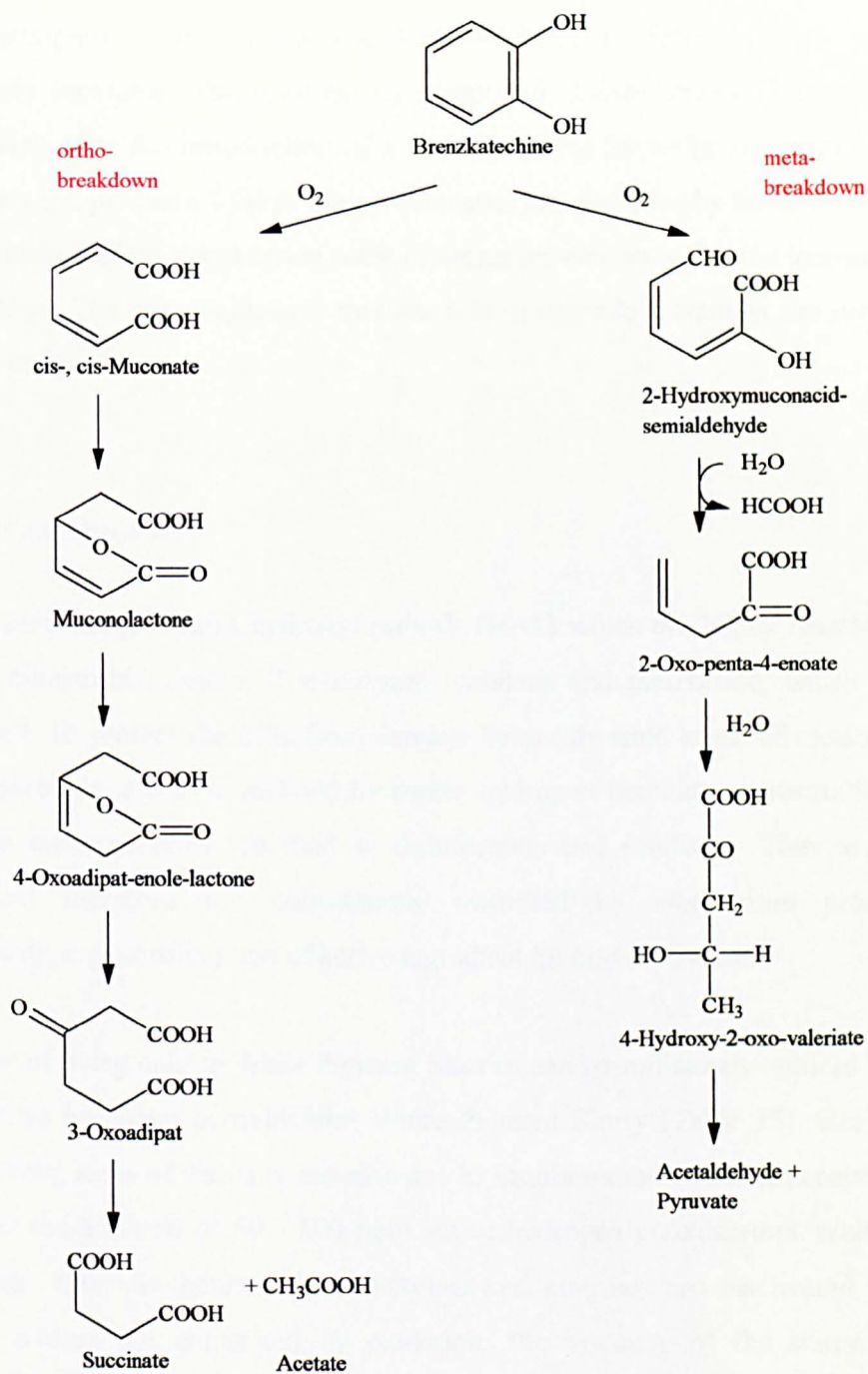


Figure 56. Degradation paths for brenzkatechines (Modified from Peter & Vollhardt, 1990).

In the meta-path, 2-hydroxymuconacid-semialdehyde is formed, from which formate is hydrolytically separated. The resulting C<sub>5</sub> compound, 2-oxopentolate, becomes accessible to an aldol cracking after the introduction of a hydroxy group by water accumulation which leads to acetaldehyde and pyruvate. Mainly methyl aromatics are degraded by the meta-pathway.

The final product of the degradation paths of aromatics can enter into the intermediary metabolism of the bacteria. The microorganisms that are able to degrade aromatics can use the substrates as sole C and energy sources.

#### 4.4.9 Hydrogen Peroxide

Hydrogen peroxide generates hydroxyl radicals (H-O<sup>•</sup>) which are highly reactive and responsible for their antimicrobial action. The enzymes catalase and peroxidase, which are produced by respiring cells to protect the cells from damage by steady-state levels of metabolically generated hydrogen peroxide, are overwhelmed by higher hydrogen peroxide concentrations, e.g. 3 – 6 % (v/v). Such concentrations are used in disinfectants and sanitisers. They are effective within minutes and therefore not considerably disturbed by inactivation processes occurring simultaneously, e.g. consumption of active ingredient by organic matter.

The number of living cells in White Pigment Slurries can be sufficiently reduced by the addition of 50 ppm active hydrogen peroxide/dmt White Pigment Slurry (Table 35). Heavily contaminated slurries showing signs of viscosity increase due to instabilisation of the dispersant and greying may be saved by the addition of 50 - 100 ppm active hydrogen peroxide/dmt White Pigment Slurry with stirring. After 24 hours, when microbes and enzymes are inactivated and most of the unpleasant odours are eliminated by oxidation, the viscosity of the slurry is restored with additional sodium hydroxide in order to increase pH and restabilise the dispersant and by the incorporation of a suitable preservative for protection.

The antimicrobial activity and decomposition of hydrogen peroxide increases and accelerates with increasing temperature. It also has to be considered that hydrogen peroxide quickly decomposes in alkaline media, i.e. calcium carbonate slurry.



HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	10 ppm <sup>1)</sup>	20 ppm <sup>1)</sup>	50 ppm <sup>1)</sup>	100 ppm <sup>1)</sup>	250 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	> 10 <sup>5</sup>	< 10 <sup>3</sup>	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>

<sup>1)</sup> The concentrations of hydrogen peroxide were calculated as active on dmt slurry.

Table 35. Minimum growth inhibitory concentration of hydrogen peroxide.

#### 4.4.10 Sodium Hypochlorite

Sodium hypochlorite solutions are appropriate to control a wide spectrum of microorganisms. Its stability is influenced by light, temperature, concentration and pH. Since the stability is higher in the alkaline range, the commercial solutions are mostly buffered with borate and have a pH value of around 12. It does not leave residuals, is virtually colourless, non-staining, easy to handle and most economical in use. Hypochlorite solutions are proven sanitisers used in households, public buildings, the food industry and hospitals. Water treatment is another application field for sodium hypochlorite.

Its use for the disinfection of White Pigment Slurries, however, involves some risks. Since sodium hypochlorite is a strong oxidising agent there is an increased risk of corrosion of tank or pipe materials if it is used over an extended period of time. The quantities which have to be used to obtain a bactericidal effect in White Pigment Slurries are shown in Table 36.

HC 90-ME 75%	TVC (cfu ml <sup>-1</sup> )					
	Control	5 ppm <sup>1)</sup>	10 ppm <sup>1)</sup>	25 ppm <sup>1)</sup>	50 ppm <sup>1)</sup>	100 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	2.1 x 10 <sup>4</sup>	< 10 <sup>3</sup>	< 100	< 100	< 100
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>

<sup>1)</sup> The concentrations of sodium hypochlorite were calculated as active on dmt slurry.

Table 36. Minimum growth inhibitory concentration of sodium hypochlorite.

4.4.11 Tetrakis(hydroxymethyl) Phosphonium Sulphate (THPS)

THPS is a quaternary phosphonium salt which releases formaldehyde. It is claimed to be effective against algae, fungi and bacteria and recommended for use in badly fouled cooling systems and injection water for secondary oil recovery as a slimicide which is especially active against sulphate reducing bacteria.

The use of THPS for the disinfection of White Pigment Slurries is not recommended. Large scale trials at the production site in Gummern (Austria) showed an incompatibility, especially when adding it to calcium carbonate slurry, where the formation of agglomerates could be seen.

THPS is more useful for the disinfection of the water cycle in a White Pigment Slurry production site. Here, however, it has clearly to be considered that THPS will be deactivated in the presence of high concentrations of oxidisers, oxygen scavengers and amines (Albright & Wilson Ltd., 2001).

For the disinfection of recycled water in a White Pigment Slurry production or at a paper mill 75 - 100 ppm THPS per t water show good bactericidal effects (Table 37).

Process Water	TVC (cfu ml <sup>-1</sup> )					
	Control	25 ppm <sup>1)</sup>	50 ppm <sup>1)</sup>	75 ppm <sup>1)</sup>	100 ppm <sup>1)</sup>	150 ppm <sup>1)</sup>
1 <sup>st</sup> inoculation	> 10 <sup>6</sup>	3.9 x 10 <sup>4</sup>	1.4 x 10 <sup>3</sup>	50	< 10	< 10
2 <sup>nd</sup> inoculation	> 10 <sup>6</sup>	-	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>	> 10 <sup>6</sup>

<sup>1)</sup> The concentrations of THPS were calculated as active on ton process water.

Table 37. Minimum growth inhibitory concentration of THPS.



#### 4.5 Adaptation, Resistance and Selection

The existence of antibiotic-resistant bacteria has been known for many years and has been comprehensively described.

In contrast, less is known about the genetic and biochemical basis of resistance to other antibacterial agents such as disinfectants and preservatives. Nevertheless, in recent years there has been a gradual increase in our knowledge of resistance mechanisms to non-antibiotic agents, and sufficient information has now been gathered to allow consideration of the topic as a separate issue from antibiotic resistance.

There are two broad categories of resistance:

- intrinsic resistance
- acquired resistance

The term 'intrinsic resistance' is used to imply that inherent features of the cell are responsible for preventing antimicrobial action and to distinguish this situation from acquired resistance, which occurs when resistant strains emerge from previously sensitive bacterial populations, usually after exposure to the inhibitor concerned (Hancock & Nicas, 1984 and Chopra, 1987).

#### 4.6 Conclusions

The increasing sensitivity of the paper and paint industries with respect to the use of disinfectants and preservatives significantly reduces the choice of possible bactericidal active agents for use in White Pigment Slurries. In the medium or long term, products which release formaldehyde and/or are sensitising will no longer be accepted or allowed due to the high risk to humans and the environment. Furthermore, it has to be remembered that the range of potential active agents is limited by the high demands placed on their physical properties such as stability to temperatures of up to 60°C or pH stability in the alkaline area.

New active agents cannot be expected to be available in the short or medium term. The new Biocidal Products Directive 98/8/EC (BPD) controls the placing on the market of biocidal products by introducing a scheme for mutual authorisation. Data requirements are extensive and consequently the cost of generating and providing this data is high.

The use of o-Phenylphenol (OPP) as a preservative in White Pigment Slurries, the focus of most of this thesis, fulfils all of the present requirements. Fast bactericidal effects, good preservative performance, excellent toxicological properties (i.e. non-formaldehyde releaser, not sensitising) as well as thermal stability at temperatures above 60°C make this active agent very interesting. The development of OPP neutralised with KOH (o-Phenylphenol potassium salt / KOPP) to replace the "commercial", NaOH-neutralised, form enabled the concentration to be increased from 20 % active OPP to 45 % active OPP (Schwarzentruher & Buri, Patent # 01943291.3-2103-EP0104729), resulting in easier handling of the product and a significant reduction in the transport costs. Only the poor curative action of OPP has to be improved. This can be achieved by combining OPP with BIT or MIT (section 6.2.3) which also reduces the risk of adaptation of microbes to the active agent OPP.

For the first time, the CellFacts I and CellFacts II technologies were used to gain a better understanding of the mode of action (see also sections 3.4 and 3.5). Based on the experimentally generated data it is possible to draw conclusions about the quantities of biocide that have to be dosed for the preservation of White Pigment Slurries. Thermal stability as well as stability in the alkaline area are criteria that should not be underestimated in the selection of suitable disinfectants/preservatives. The use of faster analytical systems such as CellFacts II makes real-time measurement of bactericidal efficiency possible, thus minimising the risk of formation of resistance of microbes to an active agent or the selection and enrichment of a different species.

## **CHAPTER 5**

### **House-Keeping**

## 5.1 Introduction

The requirements for the delivery of preserved slurried products begins from the moment the mineral is extracted or synthetically produced. The process conditions are as important regarding bacterial contamination and control as to the delivery and storage strategy of the end-product itself. An environment of "good housekeeping" is essential in optimising the microbiological control needed for preservation and acceptable application of the pigment in its end-use.

During the fine grinding processes, for example, temperatures of up to 110°C can be reached. This ensures to a large extent the thermal disinfection of the processed mineral. Thus, there generally occurs a significant reduction of the bacterial count, arising initially from contaminated plant and treatment waters and process additives, from that found in the feed material. After such a grinding or high temperature processing step, dispersions can often be regarded in microbiological terms as of pharmaceutical quality. However, there are some cells which, due to a certain protection mechanisms, that are not destroyed by the grinding process but are "transferred" into a physiologically stressed or dormant state (Figure 57 & 58 (Schwarzentrubler, 2001)). During storage of slurry products, post-dispersion of synthetic products or during transportation, the temperature can decrease (or rise again) to a level which is favourable to bacterial growth (25 - 45°C). Growth can then proceed either from re-infection from outside sources, usually by fluid contamination or even contact with air, or by the recovery / reactivation of the dormant or stressed cells. It is on this post-processing contamination that focuses most attention when considering slurry preservation. Of course, should a processing step involve lower temperature handling or combinations of products then a complete revision of the process environment in respect to bacterial contamination must be made. This is specific to the plant in question and requires extensive analysis of water systems, settling and waste recovery vessels, and flash coolers (Schwarzentrubler & Gane, 2002).

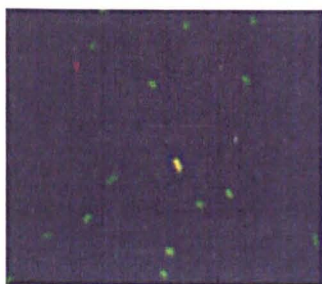


Figure 57. Bacterial physiology before fine grinding. Here, the green cells are clearly visible, i.e the cells are viable (magnification 1000x).

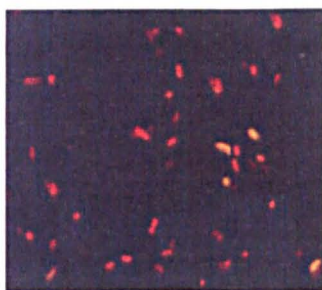


Figure 58. Bacterial physiology after fine grinding. The red cells on this picture are dead. The cells appearing orange under the fluorescent light are physiologically stressed. In this state growth is no longer possible. The cell either dies or recovers after a certain time (magnification 1000x).

It is obvious that an initial phase with a majority of dead and a relatively small amount of physiologically stressed cells increases the preventative action of the biocides, thus ensuring the long-term stability of the product.

With processing steps in which neither thermal nor chemical disinfection can be achieved there is a high risk of biofilm formation. Biofilms can continuously contaminate a system, i.e. White Pigment Slurries, and provide an environment for microorganisms to adapt.



## 5.2 Recognition of Plant Specific Parameters

The major problem in the introduction of a house-keeping strategy in production sites is the fact that practically every plant has got specific parameters which have to be considered:

- Tank level schedule varies in individual plants.
- Concrete and steel tanks coexist.
- Agitators are different with respect to form as well as speed.
- Exact addition of preservatives is difficult.
- Temperatures in tanks are different (i.e. high temperatures still offer good thermal disinfection).
- Disposal of diluted or dispersed slurry is a problem.

It is obvious that concrete tanks offer numerous cavities, pores and cracks which are predestined for the formation of biofilms. Dead corners in storage tanks can also be a rich source of continuous contamination since the distribution of microbiocides is limited. In addition to this, units for cooling the slurry are frequently used, offering beside other physio-chemical advantages better bactericidal performance of the preservative (thermal decomposition will be minimised) on the one hand, but on the other hand they also provide a "good" environment for bacterial growth. It then becomes clear that the implementation of a house-keeping protocol has to be well planned and the plant specifically adjusted.

### 5.3 Sediment and Biofilms

Biofilms are localised concentrations of microorganisms attached to a substratum and consist of a population of a single species, or more often a multi-species community. Within the biofilm, heterogeneous distributions of organisms and metabolic activities are common.

Attachment / entrapment in biofilms is advantageous for many microorganisms. The benefits include availability of nutrients concentrated at surfaces; access to a flowing system which increases the availability of diffusable nutrients; the ability to generate microniches by metabolic activities such as the generation of anaerobic sites in an aerobic environment and enhanced metabolic process by interaction within consortia.

The majority of biofilms that form on metals and plastics exhibit an open architecture, a heterogeneous structure with water channels (Figure 59). The structure typically consists of a series of microcolonies which aggregate into characteristic stacks or fronts (Keevil, 1999).

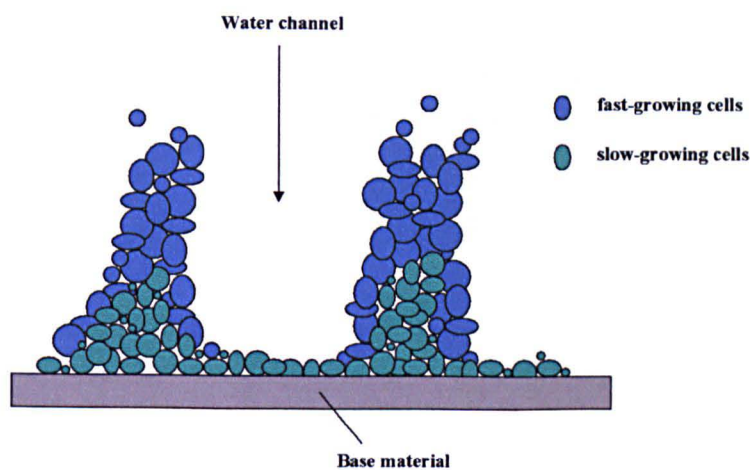


Figure 59. Heterogeneous biofilm structure with water channels.

The phenomenon of bacterial resistance to biocides in distribution systems can be ascribed to a process of adaptation (i.e. induction of genetic mechanisms) and not only to selection of resistant bacteria (i.e. intrinsic cell resistance) by organisation and composition of bacteria. Prokaryote cells respond to environmental stresses by induction of a large range of specific mechanisms. A specific set of genes activated by environmental stress constitutes a stimulon such as the heat shock regulation system, the SOS genetic system and the oxidative stress regulation system.

The formation of sediment on the bottom of a tank (Figure 60) due to insufficient mixing as well as the formation of biofilms on walls of tanks (Figure 61 & 62) and pipelines represent excellent environments for bacteria, i.e. bacteria are protected from physical and chemical impacts and challenge.



Figure 60. Sediment from White Pigment Slurry on the bottom of a storage tank.

In addition to the above, tanks are hardly ever emptied completely. It often happens that a relatively low quantity of "residual" product remains in the tank for up to 10 days or longer. During this period of time, these products are not subject to microbiological monitoring. If freshly produced material is then filled into this tank, it cannot be predicted how strongly the biocide in the new product is diluted (by the old one) and whether there are already microbes in the old product which could be a source of contamination.



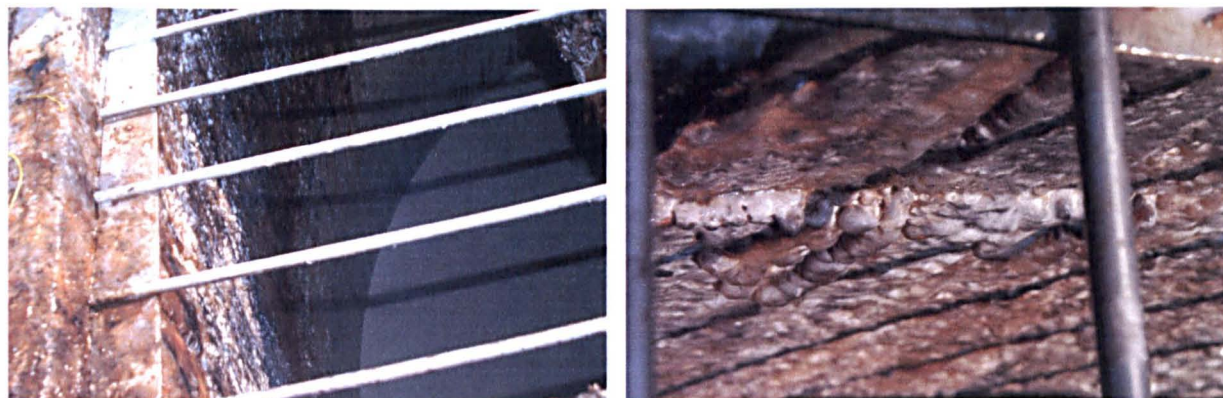


Figure 61 & 62. Biofilm on the wall of a White Pigment Slurry storage tank at a paper mill.

At increased risk are tanks which are "empty" but contain, for example, a  $\text{CaCO}_3$  sediment which makes growth of bacteria easier and, to some extent, protects them from the biocides so that, in the worst case, resistance to biocide can be built up.

To be able to prevent the formation of resistance and a further increase of the biocide quantity, it is essential to remove sediments and biofilms from the storage tanks.

### 5.3.1 Formation of Resistant Bacteria

A number of factors must be considered in choosing a biocide for an individual treatment. The biocide(s) must be active against the bacteria in the system under *in situ* conditions and must remain active for a long enough period of time to treat sites far removed from the injection site of the chemical. The biocide must be compatible with any other chemical agents that may be used such as dispersant agents, flotation agents. Lastly, the biocide must be economic.

This is exactly the difficulty in preventing the formation / enrichment of resistant bacteria. On adding a bactericidal substance it has to be made sure that the concentration is lethal, i.e. above the MIC. Continuous underdosing as well as doses that are strictly at the limit of the MIC involve the risk of a formation of resistant bacteria.

As already mentioned in section 5.3, biofilms as well as sediments provide bacteria with optimum protection against bactericidal substances. Often, only small quantities of biocides can reach these bacteria and be absorbed by them which means that "familiarization" can occur in the course of time.

The physiological state of a cell should not be underestimated either. If the cells are in a physiologically stressed or compromised state they will not absorb the biocide or will do so only in non-lethal quantities due to the suppressed metabolism. Again, there is the risk that familiarization of the cells will occur.

To minimise the formation of resistant bacteria the use of an efficient biocide in bactericidal quantities is essential. It has to be remembered here that with two to three different bactericidal active agents a much broader spectrum of microbes can be covered, thus reducing the problem of resistance. In section 5.3.1.2 a case is described where resistance was supported by the use of one single active agent for years and the only possibility to solve this problem was a change of biocide.

To support the action and efficiency of a biocide the implementation of a house-keeping as well as a suitable and fast monitoring is very important and should not be underestimated.

#### 5.3.1.1 Spore-Forming Bacteria

In the White Pigment Industry, but mainly in the paper industry, bacterial problems are often attributed to the presence of spore-forming bacteria. However, in the many analyses which were made, neither spores nor spore-forming bacteria were found. However, studies were carried out to investigate how spore-forming bacteria behave in  $\text{CaCO}_3$  slurries.

The experiments showed that a  $\text{CaCO}_3$  slurry can serve as a growth substrate for different *Bacillus* species.



When spore-forming bacteria (e.g. *Bacillus* species) have to compete with other vegetative, Gram-negative cells their growth is impaired because of the dominance of the latter. Indeed, it was observed that the *Bacillus* became non-viable in these experiments.

This is not surprising as the poor supply of organic nutrients in a  $\text{CaCO}_3$  slurry are used up by other species. Due to this, the growth of *Bacillus* species as well as the presence of spores ( $> 100 \text{ cfu ml}^{-1}$ ) in a  $\text{CaCO}_3$  slurry is much reduced. This fact has been confirmed by the practical experience of many years.

Under the experimental conditions chosen, even a concentration of 10 ppm of the active agents formaldehyde and isothiazoline-one showed a bactericidal effect against the *Bacillus* isolates.

The survival of bacteria in the order of  $100 - 200 \text{ cfu ml}^{-1}$  in the samples containing biocide can be explained by the possibility spores were present in the inoculum and these were resistant to the quantities of biocide used.

Test bacilli:       - *Bacillus cereus* DSMZ 31  
                      - *Bacillus subtilis* DSMZ 347  
                      - *Bacillus* isolate from „Pink Slurry“ ex Orgon

Pigment slurry:   HC 90-GU 75%, produced and pasteurised on 07.03.2000

Experimental procedure:   24 hours agar cultures (tryptic soy agar) were suspended in physiological sodium chloride solution with 0.1% (w/v) peptone. The cell densities were adjusted to  $6.0 - 6.5 \times 10^6 \text{ ml}^{-1}$  (CellFacts I) and checked by plating on PC agar. 50 g samples of slurry were inoculated with 0.2 ml each of the bacterial suspensions and incubated at  $30^\circ\text{C}$ . After three and seven days, the colonies were counted.

<i>Bacillus</i> species	cfu ml <sup>-1</sup> Pigment Slurry		
	after Inoculation	after 3 Days	after 7 Days
<i>Bacillus cereus</i>	2.8 x 10 <sup>3</sup>	1.7 x 10 <sup>4</sup>	5.2 x 10 <sup>5</sup>
<i>Bacillus subtilis</i>	1.8 x 10 <sup>3</sup>	1.6 x 10 <sup>5</sup>	7.8 x 10 <sup>5</sup>
Isolate „Pink Slurry“	1.2 x 10 <sup>3</sup>	3.4 x 10 <sup>4</sup>	8.6 x 10 <sup>5</sup>
<i>B.cereus</i> / <i>B.subtilis</i> + pool of 4 Pseudomonad isolates	5.8 x 10 <sup>3</sup>	7.9 x 10 <sup>2 a)</sup>	5.0 x 10 <sup>2 a)</sup>
<i>B.cereus</i> / <i>B.subtilis</i> + pool of 2 Pseudomonad isolates	6.8 x 10 <sup>3</sup>	3.1 x 10 <sup>3 a)</sup>	8.9 x 10 <sup>2 a)</sup>
<i>Bacillus cereus</i>	5*	1.2 x 10 <sup>3</sup>	1.1 x 10 <sup>4</sup>
<i>Bacillus subtilis</i>	6*	1.2 x 10 <sup>3</sup>	4.1 x 10 <sup>4</sup>
Isolate „Pink Slurry“	2*	5.0 x 10 <sup>5</sup>	3.2 x 10 <sup>5</sup>
<i>B.cereus</i> / <i>B.subtilis</i> + pool of 5 Pseudomonad isolates	23*	< 10 <sup>a)</sup>	< 10 <sup>a)</sup>
<i>B.cereus</i> / <i>B.subtilis</i> + pool of 2 Pseudomonad isolates	19*	< 10 <sup>a)</sup>	< 10 <sup>a)</sup>

\* calculation based on the density of the inoculation suspension

<sup>a)</sup> spores

Table 38. Evaluation of the ability of *Bacillus* species to grow in CaCO<sub>3</sub> slurries.

Concentration Biocide AK	cfu <i>Bacillus</i> ml <sup>-1</sup> CaCO <sub>3</sub> Slurry
10 ppm	< 100
20 ppm	100
50 ppm	200
100 ppm	< 100
Control without biocide	6.0 x 10 <sup>5</sup>

Table 39. Evaluation of the effectiveness of active agents against *Bacillus* species in CaCO<sub>3</sub> slurries.

### 5.3.1.2 Facultative Methylotrophs

When biocides based on pure formaldehyde are used, microorganisms which, for example, use formaldehyde as a carbon source, can acclimatise in White Pigment Slurries in the course of time and thus cannot be eradicated with pure formaldehyde biocides.

This fact can heavily impact on the preservation of filler and pigment slurries, in some cases an optimum preservation may even be rendered impossible. In this case, changing to another active agent or using a combination of active agents is the only remedy.

This was exactly the problem the Omya production site in Orgon (France) had been encountering in its observably pink slurries and was due to the lack of a house-keeping protocol where microorganisms became accustomed to the biocide by protection in sediments and/or biofilms.

Methylotrophic microorganisms have specialised in the aerobic use of  $C_1$  compounds. The expression methylotrophic describes the ability to use methanol. However, other  $C_1$  compounds are also used by these organisms as an energy and carbon source. The majority of the methylotrophic bacteria are obligately methylotrophic, meaning that they can only grow on  $C_1$  compounds. Typical examples of obligately methylotrophic bacteria are:

- *Methylomonas*
- *Methylomicrobium*
- *Methylobacter*
- *Methylococcus*
- *Methylosinus*
- *Methylocystis*
- *Methylophilus*
- *Methylobacillus*

Facultative methylotrophs can, apart from  $C_1$  compounds, also use complex substrates. Among the  $C_1$  compounds, methanol, formaldehyde, formate and methylamine are used, but not methane itself (Hood & Dow & Green, 1988).

Typical examples of facultative methylotrophic bacteria are:

- *Methylobacterium*
- *Hyphomicrobium*
- *Acetobacter*
- *Arthrobacter*
- *Mycobacterium*
- *Paracoccus*- and *Nocardia*-species

Some methylotrophic bacteria can use not only methanol but also methane as they have the enzyme methanomonooxygenase which is necessary for its oxidation (Figure 63).

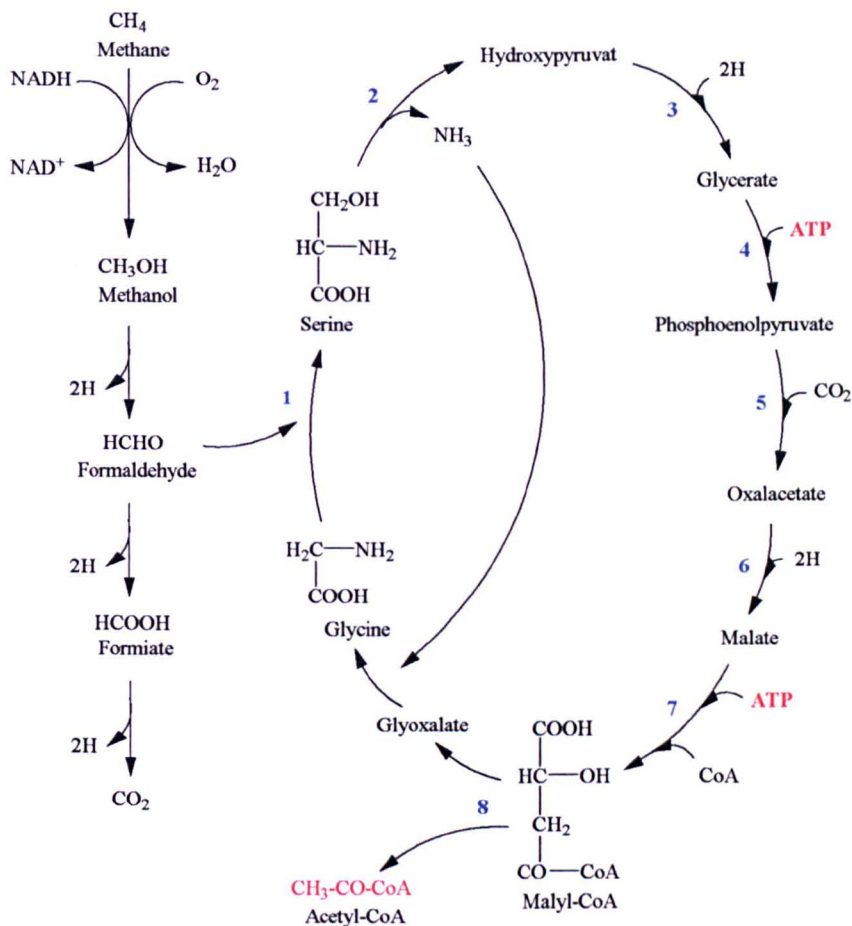


Figure 63. Methane metabolism in the methylotrophic bacteria. Assimilation of  $\text{C}_1$  compounds through the so-called serine cycle.

As already mentioned in section 3.3.3.2 direct removal and concentration of bacteria from the investigated calcium carbonate slurries from Orgon by differential centrifugation, followed by light microscopy, revealed much greater morphological variation than would be expected. Also, plating on reduced arginine starch salts (RASS) medium as well as on agar plates containing ethylene glycol hemiformal showed greater diversity.

The Orgon slurries, which were investigated due to signs of resistance to a formaldehyde based biocide, have proved to be a rich source of microbial diversity with *Rhizobium*, *Agromyces* and the facultative methylotroph *Methylobacterium* species all being isolated (in addition to *Pseudomonas* species), and *Hyphomicrobium* species being detected by microscopy (Figure 64 & 65).



Figure 64. Phase contrast micrograph of *Hyphomicrobium* species isolated from calcium carbonate slurry ex Orgon (magnification 1000x).



Figure 65. *Methylobacterium* isolated from calcium carbonate slurry ex Orgon and cultivated on PCA containing ethylene glycol hemiformal.



The efficacy of biocides to facultative methylotrophs was studied:

- Biocide/Disinfectant: - Biocide A  
 - Biocide AK  
 - Biocide ABK  
 - Preventol OF 45

50 g of OMYAFIL 60 were inoculated with the pink pigmented bacteria isolates from the "Pink Slurry" (microbial load =  $1.2 \times 10^6$  cfu ml<sup>-1</sup>). Subsequently, different concentrations of biocide were added. After 24 hours and 4 days of incubation at 30° C, respectively, the bacteria were counted (Table 40).

Biocide	ppm (active on dry)	cfu ml <sup>-1</sup> aerobic methylotrophic bacteria	
		24h after inoculation	4d after inoculation
Biocide A	100	$3.0 \times 10^6$ <sup>1)</sup>	$5.0 \times 10^6$ <sup>1)</sup>
	200	$8.3 \times 10^5$ <sup>1)</sup>	$1.3 \times 10^7$ <sup>1)</sup>
	500	$4.3 \times 10^5$ <sup>1)</sup>	$2.9 \times 10^6$ <sup>1)</sup>
Biocide AK	100	< 100	< 100
	200	< 100	< 100
	500	< 100	< 100
Biocide ABK	100	< 100	< 100
	200	< 100	< 100
	500	< 100	< 100
Preventol OF 45	100	< 100	< 100
	200	< 100	< 100
	500	< 100	< 100
Control	-	$1.3 \times 10^7$	$8.2 \times 10^6$

1) The species was identified as a *Methylobacterium extorquens*.

Table 40. Efficacy of biocides to facultative methylotrophs.

The study clearly revealed that the microorganisms resistant to formaldehyde could be eradicated by adding a further active agent or even exchanging one active agent for another.

In a second experiment the pink pigmented bacteria were transferred onto plate count agar which contained various concentrations of formaldehyde (50 - 200 ppm). After 48 hours incubation at 30°C, it was possible to observe growth on all plates. This is also an indication of the presence of pink pigmented facultative methylotrophs.

As mentioned above, these species need substances such as methanol or formaldehyde as a carbon source. Over a long period of time Orgon was only using biocides based on pure formaldehyde and clearly did not establish a house-keeping protocol. It can be assumed that these species have built up over the years.

Characterisation of the microbial diversity of environmental samples by analysing rRNA sequencing data is a well established technique and has a number of advantages over traditional plating procedures. The main advantage of the technique when utilised fully is the removal of all cultivation/isolation steps while permitting the detection and identification of individual species in a mixed population.

To date the application of the technique to White Pigment Slurries has been restricted to PCR amplification and sequencing of 16S rRNA from slurry isolates – largely in response to the limited identification offered by the API identifications system.

Table 41 Bacterial isolates from calcium carbonate slurry ex Orgon identified from 16s rRNA sequencing data:

Bacterial Isolate	Source	Base Pair Alignment (% similarity)
<i>Pseudomonas stutzeri</i>	Orgon	96
<i>Methylobacterium extorquens</i>	Orgon	96
<i>Rhizobium sp.</i>	Orgon	95
<i>Agromyces sp.</i>	Orgon	95

It is interesting to note that a number of species isolated and identified from 16S sequence data only show 95% homology (direct similarity) to those contained in the database (95% similarity is taken as „safe“ for genus level identification, while 98% and above is accepted as „safe“ to species level). This adds evidence to the idea that many of the organisms found in slurries may be fairly unique or unusual organisms, being far removed from type strains and known isolates. The 16S data also reveals, perhaps not surprisingly, that most of the isolates typified to date are common soil and water organisms.

The use of biocides based on pure formaldehyde at Orgon allowed observably pink-pigmented facultative methylotrophs to establish over a period of time. The slurry itself showed a distinctive pink gleam.

The active substances tested acted quite differently. Biocide A showed no killing effect against both the pink pigmented bacteria as well as against at least one of the gram-negative bacteria, whereas Biocide AK, ABK and Preventol OF 45, even at concentrations of 100 ppm, were able to kill all vegetative bacteria.

The consequence was that the biocide had to be changed at the Orgon plant. The Biocide A which was in use was no longer sufficient and involved the risk of the re-enrichment of facultative methylotrophs.

#### 5.4 Recommendation for Engineering

This thesis has clearly shown the requirement for a strong house-keeping regime. For the optimisation of the use of preservatives and assurance of the microbiological stability of White Pigment Slurries an effective house-keeping program is essential.

To ensure successful application of such a concept, it is necessary to check and optimise the technical circumstances in the individual production sites and storage facilities.

Some examples are:

- epoxy coating of concrete tanks in order to prevent porosity and to minimise the formation of biofilms
- conical bottoms in the storage tanks in order to reduce the formation of sediments (such bottoms will enable the operator to easily remove sediments)
- efficient agitators (good mixing will ensure fast biocide distribution into every part of the tank as well as prevent settling effects)

This is only a selection of possibilities which facilitate good house-keeping. Experience has already shown that implementation of tank cleaning twice a year as well as the use of a fast monitoring systems (such as CellFacts II) has led to a reduction of the biocide costs in a plant of upto 20% per year.

In Section 5.5, an example is given of how a continuous source of contamination can be identified and then eliminated through good "house-keeping".

### 5.5 Procedural Recommendations

In a study carried out in Orgon (France), flash cooler systems were found to be a significant source of contamination. After grinding in the PX the slurries are clean ( $< 100 \text{ cfu ml}^{-1}$ ), whereas after the flash cooler they show a bacterial count of  $> 10^4 \text{ cfu ml}^{-1}$ . These results were confirmed in the Gummern plant (Austria). Investigations showed that contamination exists and even biofilms can be formed in the flash coolers. In addition, with biofilms there is the risk of adaption (formation of resistance) of microorganisms.

This section presents strategies for the disinfection of the different Flash Cooler Systems used at Omya plants. The concepts are deliberately kept general as they have to be adjusted to each plant individually. The periodic disinfection of the Flash Coolers has been shown to lead to an increase in the performance of the biocides since only a minor curative action is required, and only preservation is needed.

As discussed in section 3.3.3 mainly mesophilic bacteria occur in white pigment slurries. The optimum growth of mesophilic bacteria is at temperatures between 25 and 45°C. If a culture of mesophilic bacteria is heated to a lethal temperature, preferably  $> 60^\circ\text{C}$  the individual cells loose viability (Figure 66).

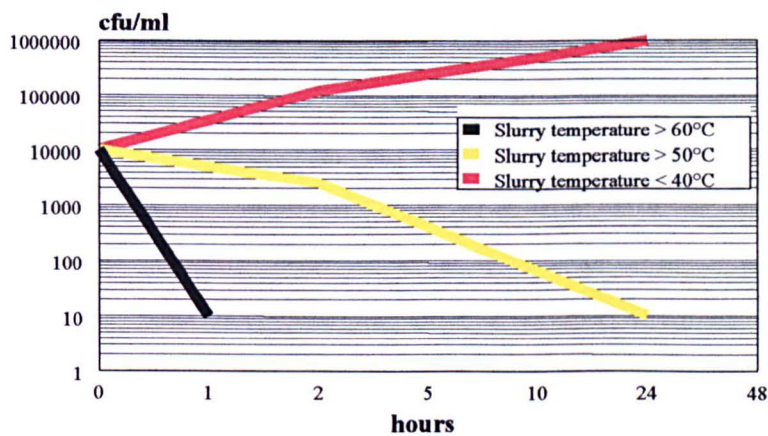


Figure 66. Thermal disinfection - lethal temperature where mesophilic cells start decaying.



Sufficient thermal disinfection of White Pigment Slurries can be achieved at temperatures of approximately 80°C.

Technical Background - Standard Operation

Slurry enters the flash chamber at about 85 - 90°C and is cooled to 40 - 50°C through sudden water evaporation (Figure 67). The temperature in the vessel depends on the actual pressure. In the heat exchanger the evaporated water is cooled and condensed. A pump discharges this condensate via spray nozzles into the steam pipe; the vacuum pump discharges leaked air and air from the slurry.

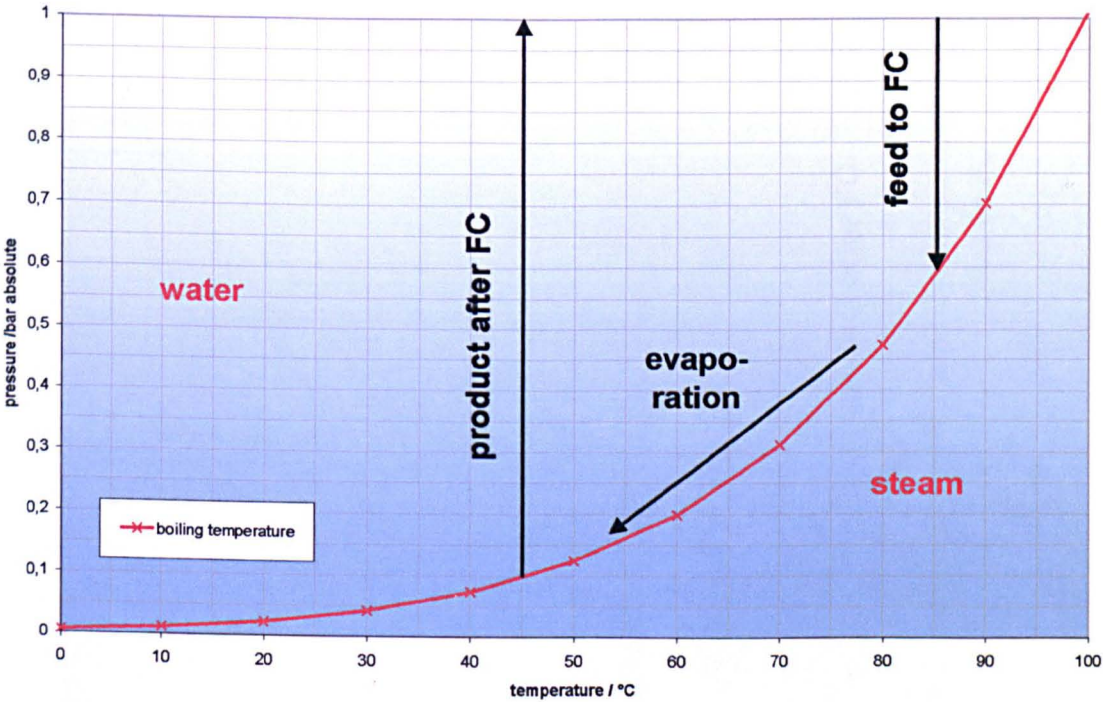


Figure 67. Temperatures of a typical standard FC process.

If the evaporated water cannot be condensed (bad operation of the cooling system) the vacuum pump is not able to maintain the vacuum and the pressure (and temperature) in the flash chamber increases. This forms the principle for the FC disinfection.

Principle used for disinfection:

After stopping the cooling system (stopping the fans) and closing a valve in the cooling circuit, the vacuum breaks down and the temperature increases. The vacuum pump stays in operation and sucks the reduced amount of steam through the steam pipe to the heat exchanger. An air inlet valve protects the vacuum pump against cavitation. Typical temperatures during disinfection are shown in Figure 68. High temperatures can be reached in the whole vessel, steam pipe and heat exchanger.

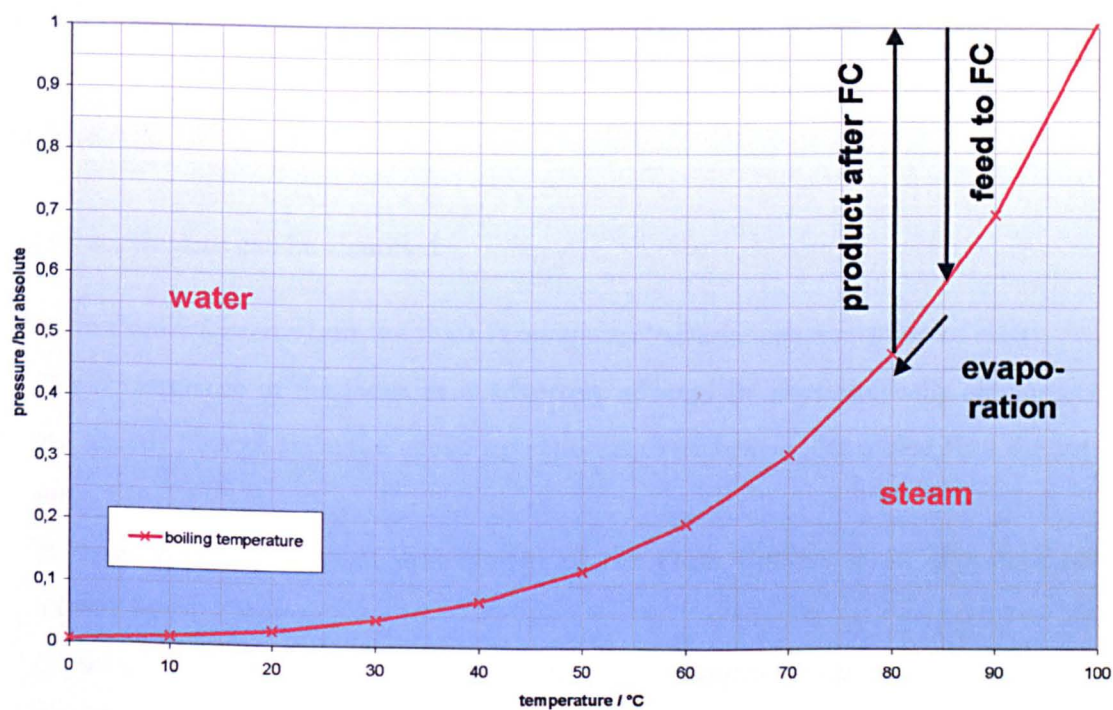


Figure 68. Temperatures of a typical FC disinfection.

With the above mentioned method, the flash vessel, steam pipe and heat exchanger could be heated up to a temperature of approx. 80°C. The duration of the disinfection cycle was between 1.5 and 3 hours.

## Results

During the first disinfection trials, contamination of the slurry product could not be reduced to  $<100 \text{ cfu ml}^{-1}$ , the cleaning water (plant water) that was sprayed to the ceiling of the flash chamber was suspected to be the source of contamination and was eliminated (turned off) during further tests.

After eliminating the source of recontamination, the contamination in the slurry product could be reduced to  $<100 \text{ cfu ml}^{-1}$ .

In these tests, a heating cycle of 1.5 hours proved to be sufficient to reduce contamination to  $<100 \text{ cfu ml}^{-1}$ . However, recontamination of the flash vessel occurred within a few days.

## Conclusion

Several conclusions can be identified:

- Flash Cooler Systems bear the risk of continuous bacterial contamination of slurry.
- The performance of the biocides is adversely affected by physiologically active bacteria that are already present since the inhibitory action is more heavily demanded than the preservative one.
- Frequent cleaning (thermal disinfection) of the Flash Coolers is an important part of the internal house-keeping.
- Cleaning the Flash Cooler Systems leads to an increase of the preservative action of the biocide, i.e. biocide costs can be optimised.
- The formation of biofilms by bacteria accumulating on surfaces and the risk of microorganisms forming resistance can be minimised.

## 5.6 Conclusions

To keep the microbiological activity in White Pigment Slurries under control and to be able to optimise the bactericidal properties of disinfectants/preservatives it is essential to implement a sound house-keeping protocol.

Due to different plant specific parameters and/or production processes, the respective protocols have to be adjusted to the individual plants.

Sediments as well as biofilms provide an excellent environment for microbes. The bactericidal action of disinfectants/preservatives can clearly be reduced, in some cases even adaptation / selection was detected (i.e facultative methylotrophs). If sediments and biofilms in storage tanks and transport facilities are removed mechanically, the production sites can use a disinfectant/biocide for a longer period of time. A change of biocide should normally be avoided since possible alternatives are limited due to the increasing limitations in the area of environment and safety legislation. It has also to be mentioned here that a change of biocide usually also entails higher costs.

## **CHAPTER 6**

### **Discussion**



## 6.1 Review of Results

This study resulted in new findings which are highly valuable for both, the pigment industry as well as for the paper and paint industries. By the combination of new analytical methods such as CellFacts II, which provides information on microbiological activity in real time, as well as by means of efficient, environmentally friendly biocides and an appropriate house-keeping protocol systems can be kept under control and costs can thus be optimised.

There is clearly a risk of microbial contamination in the process of the production of White Pigment Slurries after the final grinding in a PX. The increasing tendency towards using closed water circuits results in more bacterial cells being introduced into the system, which cannot completely be eliminated by the thermal disinfection during fine grinding since they have already developed protective mechanisms (deceleration of metabolism, formation of slime layers). The longer retention times in storage tanks as well as the duration of transport to the customers make increased cell activity in the product a serious problem.

To optimise the action of biocides (cells are more sensitive in the exponential phase of growth) early detection of microbiological activity is essential. The use of CellFacts II makes it possible to obtain information on the physiological state of bacteria nearly in real time. The use of this new technology enables, for the first time, a better understanding of the effectiveness and mode of action of preservatives to be obtained.

The use of suitable preservatives in the pigment, paper and paint industries is increasingly complicated. Regulatory, health, safety and environmental issues reduce the choice to a minimum. Clearly noticeable is the trend towards abstaining from using formaldehyde-releasing products or products with a sensitising potential (R43 labelling) in the paper and paint industries. New, innovative biocides cannot be expected in the short or medium term. The high costs of registration and evaluation with respect to the new Biocidal Product Directive (BPD) constrains the biocide industry in the search for new products.

By the further development of the active agent o-Phenylphenol (OPP) a preservative fulfilling all requirements was found. It would appear that a basis for a future preservative has been identified which can be improved by further development (combination with other active agents) with respect to the curative action (section 6.2.3). Both the bactericidal as well as the physical and toxicological properties of OPP are convincing.

The implementation of an efficient house-keeping protocol should not be underestimated. Biofilms in tanks and pipes as well as sediments on the bottom of a tank are predestined to the formation of resistant bacteria. This should absolutely be minimised or avoided since, due to the reasons mentioned above, the choice of bactericidal active agents is limited.

This work did not focus on the molecular biological area. For the time being, the information of which species are present in a system is secondary. The information on the efficiency of biocides against a spectrum of microbes as well as the exact understanding of their mechanism is much more important and has to be rated higher from an economic point of view. Nevertheless, it was possible to show that the species occurring in White Pigment Slurries are generally water and soil bacteria.

## 6.2 Further Work

### 6.2.1 Fluorescence *in-situ* Hybridisation (FISH)

Although the approaches used so far have yielded a great deal of useful information regarding community composition and profile, none of them help with the quantification of contamination, and this causes the greatest commercial concern i.e. it is vital to quickly identify the level of contamination to permit greater control over biocide dosage. An ideal tool combining the molecular biology discussed so far which gives the required information quickly is fluorescence *in situ* hybridisation (FISH).

FISH has been used primarily with prokaryotic communities and allows the direct identification and quantification of specific or general taxonomic groups. The process involves fixing the cells, hybridising their 16S rDNA (in this case) with fluorescently labelled taxon-specific oligonucleotide probes, and viewing by epifluorescence microscopy. As whole cells are hybridised, artefacts arising from biases in DNA extraction, PCR amplification and cloning are avoided. FISH can detect microorganisms across all phylogenetic levels, and probes can be generated without prior isolation of the bacteria.

Figure 69 below shows nycodenz recovered  $\text{CaCO}_3$  slurry from Gummern (Austria). This micrograph is a UV filtered photograph, showing the Cy5 (coupled to a universal probe) and a red-filtered image capturing the DAPI stain, overlain with a phase contrast micrograph. The majority of cells appear purple in colour, indicating that they are dual stained with the universal probe and Cy5 (red) together with the counter-stain DAPI (blue). Phase contrast picks out the plane of focus, giving the final picture a 3-dimensional effect.

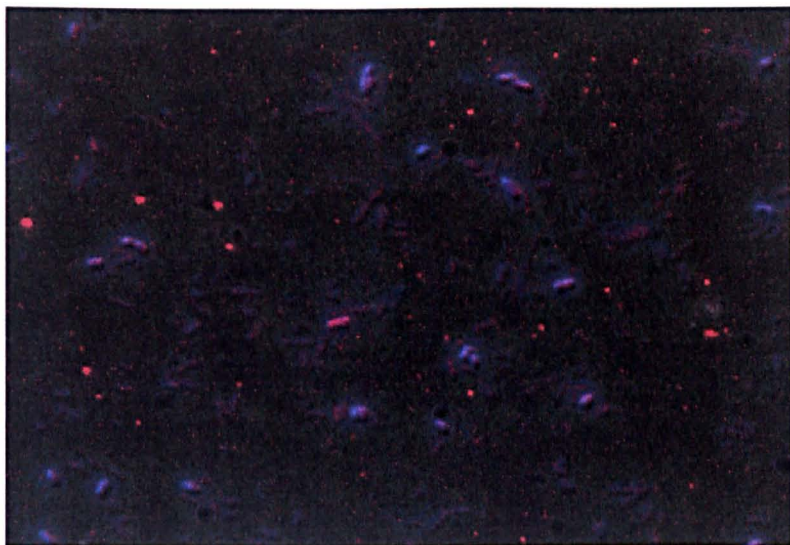


Figure 69. Nycodenz recovery from a  $\text{CaCO}_3$  slurry from Gummern (Austria), stained with Cy5 (universal probe) and DAPI, and including phase contrast.

A preliminary attempt was made at designing a probe designed for the detection of only pseudomonads, but this was found to be, even in low stringency conditions, ineffective at detecting all pseudomonads (Figure 70).

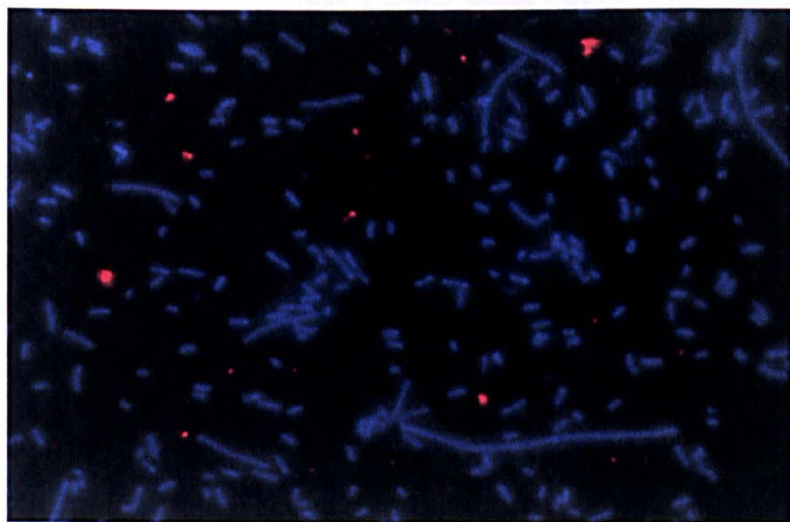


Figure 70. Pure culture of *Pseudomonas stutzeri* stained with Cy5 attached to a '*Pseudomonas* specific' probe and counterstained with DAPI.

Further probes specific to the assumed dominant species in slurry, namely *Ps. stutzeri* and *Acidovorax* are currently under design. Based on sequences retrieved from cloning experiments and published sequences, preliminary attempts have been made at designing a probe for *Ps. stutzeri* using the ClustalW program, found at [www.ebi.ac.uk](http://www.ebi.ac.uk).

However, even though these sites may be conserved within the targeted organisms, they may not be suitable probe sites due to areas of the 16S rRNA molecule being inaccessible because of structural folding. The efficiency of binding to areas within the 16S rRNA molecules have been systematically studied by Fuchs *et al.*, (1999) and suitable probe sites found by multiple sequence alignments in ClustalW may be cross checked with this to ensure their suitability. Finally, potential probes must be tested in CHECK\_PROBE in the Ribosomal Database Project (<http://rdp.life.uiuc.edu>) to ensure there is no chance of primer dimers or chimeras forming. Due to cost and time restraints, no probes specific to slurry bacteria have been fully designed and constructed. There are a number of published probes specific to *Ps. stutzeri* and *Acidovorax* that may be studied in the future (Amann *et al.*, 1996, Bennasar *et al.*, 1998). These papers highlight the difficulties of designing probes specific to the detection of pseudomonads and pseudomonad-like species, as they are a phylogenetically heterogeneous group of bacteria. Even within the species *Ps. stutzeri*, there are seven genomovars (DNA-DNA similarity groups). It may be necessary for multiple probes to be designed e.g. the use of a blocker probe (which itself could be tagged with a different coloured fluorescent marker to the main probe).

As with the other methods that have been discussed, the limitations of FISH should be considered and minimised if possible. To be detected, the microbes must be metabolically active and possess cell walls sufficiently permeable to allow penetration of the probe. Since the signal conferred is correlated to cellular rRNA content and growth rate, it is likely that slowly growing cells (possible in slurry) could be difficult to detect. In these conditions penetration of the probes into the cell can be a problem, with the cells being in a state of dormancy or quiescence, and so being smaller. It is possible, however, to add nutrients to stimulate metabolic activity, but this should always be done so as not to bias the community profile. Another alternative would be to increase sensitivity by indirect labelling, using more sensitive labels or multiple labelling (Amann *et al.*, 1995). When



using FISH to examine all members within a given taxon, one must keep in mind that the probe being used is only as good as the representative members that were used to generate it. Other, non-cultured organisms may not be detected with this probe or cross-hybridisation with related organisms may occur.

As well as using FISH in conjunction with microscopy, the technique has the potential to be used with other technologies. It is hoped that FISH can be combined with impedance flow cytometry in the new CellFacts II instruments. This will allow the real time analysis of slurries, revealing both total contamination, and eventually the detection of particular bacteria (for example those that are resistant to biocide or those that are highly pigmented and cause discoloration problems). Alternatively, fluorescently labelled probes can be used in conjunction with dot-blot hybridisation. After PCR on either isolates or nycodenz recovered slurry preparations, the product can be run on a mini polyacrylamide gel and then blotted onto a nitrocellulose membrane. Hybridisation of the probe would give a fast and accurate identification of the organism. Alternatively, the technique could be used in a similar way to screen clone libraries, cutting down on unnecessary sequencing.

It is also possible that growth within a slurry could be followed using tracking dyes. This would allow the quantification of slow growing cells that may have too little rRNA to be detected fluorescently and are therefore difficult to initially detect. Although work in this particular area has only just begun, it shows much promise, and may well become a very valuable tool for the analysis of calcium carbonate slurries.

One major disadvantage of this procedure, particularly concerning biocide efficacy, is the lack of physiological information, i.e. assessment of individual cell metabolic activity.

### 6.2.2 Real-Time Analysis of Preservative Volume

In the CellFacts II technology a method was found which enables the microbial activity to be analysed in real time. This ensures that an inactive biocidal active agent or underdosing is rapidly detected and action (i.e. additional preservation) can be taken promptly. However, this technology does not enable a possible overdosing to be detected. Since exact dosing of preservatives / disinfectants is often difficult, due to the technical circumstances, these phenomena are often noticed in the white pigment industry as well as in the paper and paint industries.

The current methods for the determination of biocidal active agents such as OPP, CIT, MIT, Bronopol and many more mainly use HPLC and are often quite time-consuming. Furthermore, the necessary infrastructure and personnel for these analyses are not available at production sites. From the point of view of economic efficiency, real-time analysis of preservative volume would considerably contribute to the optimisation of profits. The dosing units could continuously be checked and the biocide household thus be optimised.

One possibility to carry out such an analysis in real time could also be the CellFacts II technology. The addition of a fluorescent marker to the preservative / disinfectant would enable not only the TVC but also the biocide content in a White Pigment Slurry to be analysed. This would be a significant advantage since two analytical requirements of the quality assurance could be carried out using one measuring principle or instrument.

### 6.2.3 Preservative Combination with o-Phenylphenol (OPP)

As already mentioned in section 4.4.8, OPP is an excellent preservative having many physical as well as ecological advantages over the "traditional" active agents, but clearly showing a weakness in its curative action.

Due to this weakness and its limited solubility it is no longer possible for the production sites to carry out an additional preservation if microbial contamination occurs. On the one hand, the bacterial count ( $< 100 \text{ cfu ml}^{-1}$ ) can no longer be controlled and on the other hand, there is the risk of sedimentation of the active agent which can lead to considerable problems in the use of the White Pigment Slurries, mainly in the paper industry (i.e. scratches on paper).

This problem could be solved by combination with a second active agent. However, since there is a clear tendency in the paper and paints industry towards using formaldehyde-free as well as non-sensitising products, the choice of products that could be combined is quite small. Furthermore, the costs of OPP per tonne of White Pigment Slurry are quite high in comparison with other active agents and these costs would be increased by combining the OPP with an additional active agent. A combination would therefore only be meaningful if the costs were kept neutral due to a synergistic effect of the two active agents improving performance.

First studies with a combination of o-phenylphenol (OPP) and 1,2-benzisothiazoline-3-one (BIT) produced quite promising results (Table 42). Since BIT does not release formaldehyde, is not sensitising and, moreover, shows high temperature stability a combination of this substance with OPP would certainly be meaningful.

HC 90-ME 75%	TVC ( $\text{cfu ml}^{-1}$ )				
	Control	250 ppm OPP <sup>1)</sup>	200 ppm BIT <sup>1)</sup>	125 ppm OPP / 125 ppm BIT <sup>1)</sup>	150 ppm OPP / 150 ppm BIT <sup>1)</sup>
1 <sup>st</sup> inoculation	$> 10^6$	$< 100$	$< 100$	$< 100$	$< 100$
2 <sup>nd</sup> inoculation	$> 10^6$	$< 100$	700	100	$< 100$
3 <sup>rd</sup> inoculation	$> 10^6$	$< 100$	$4.9 \times 10^5$	$3.6 \times 10^4$	$< 100$

<sup>1)</sup> The concentrations of OPP and BIT were calculated as active on dry metric ton (dmt) slurry.

Table 42. Minimum growth inhibitory concentration of OPP / BIT formulations.

## **CHAPTER 7**

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## **CHAPTER 8**

### **Publications**



## Publications

1. Schwarzentruher, P., Gane, P.A.C., 2003. Application of Microbiocides for the Storage Protection of Mineral Dispersions. In Paulus, W., Directory of Microbicides for the Protection of Materials and Processes. Dordrecht: Kluwer Academic Publishers.
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## **Application of Microbiocides for the Storage Protection of Mineral Dispersions**

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## Introduction

The subject 'Application of Microbiocides for the Storage Protection of Mineral Dispersions' is of ever-increasing interest for scientists and industrialists and includes many challenges for the mineral slurry producer and user. Increasing conversion from dry pigment handling to water-based dispersions is taking place over a wide range of production applications, for example, papermaking filler products and coatings formulations in both the paper and paint industries.

The requirements for the delivery of preserved slurried products begins from the moment the mineral is extracted or synthetically produced. The process conditions are as important regarding bacterial colonisation and control as the delivery and storage strategy of the end-product itself. This article attempts to give a brief insight into the background issues and procedures needed to provide an environment of "good housekeeping", essential in optimising the microbiological control needed for preservation and acceptable application of the pigment in its end-use. On this base, the latest research on the bacterial strains, their identification, measurement and colony growth dynamic is presented, and the biocide strategies, applicability and constraints are discussed. Illustrations are given throughout of the sources of microbiological contamination likely to occur during production, storage and transportation.

Based on the current knowledge being gained from combining active Research and Development and on the ground Applications expertise, new possibilities for optimising microbiological quality control are described.

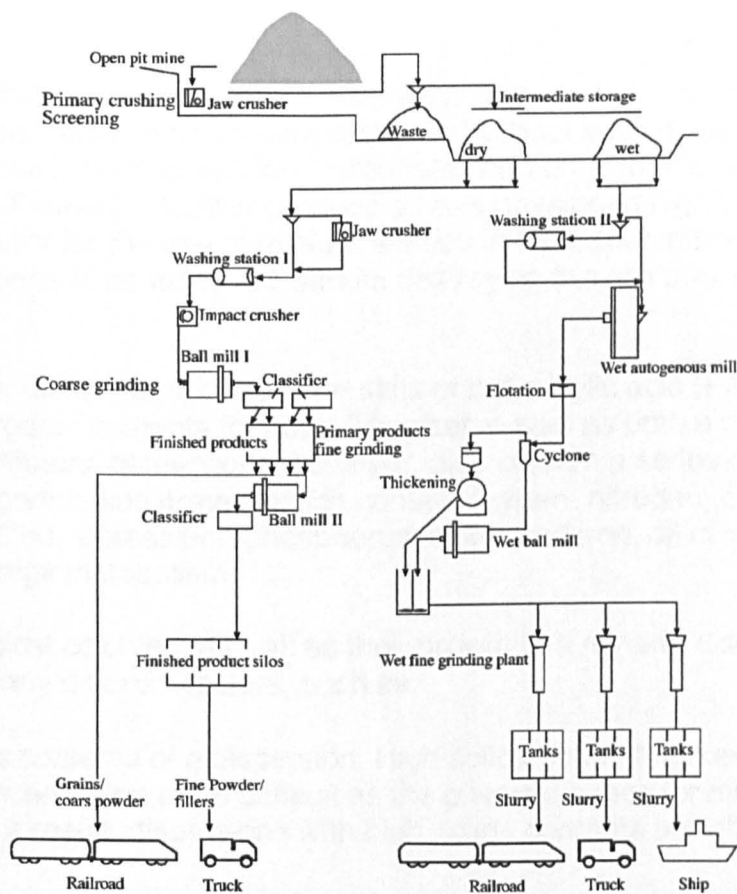
## 1. Pigment manufacturing process

From the mining of raw stone to the delivery of a pigment suspension to the paper industry, the material passes through a wide range of processing steps and procedures, typically as might be seen schematically in figure 1. Following the breaking of the mineral or mineral-containing stone, the first crushing step, the material is usually washed and prepared for optical and size selective sorting. To separate unwanted finely intra- and inter-grown minerals, beneficiation by flotation is often carried out prior to further more stringent size classification by hydrocyclone or centrifuge. If synthetic pigments are to be made, the raw material, such as limestone, prior to burning or calcining, must also be carefully selected. The slaking process for precipitated calcium carbonate, for example, requires specific control of burnt lime sourcing and particle size before the carbon dioxide addition stage.

Adjusting the parameters of wet (waterborne) fillers or pigments to the respective requirements of paper making and coating, is achieved by control of crystal growth, in the case of precipitated products such as precipitated calcium carbonate or precipitated silica, control of particle delamination and comminution, in the cases of kaolin, mica and talc, or by grinding and selection technology in a wet milling process, for materials such as ground calcium carbonate. Each methodology is pursued at a controlled solids content in either a dispersed or a flocculated state, depending on the mineral base and the use of various dispersants and flocculants. Final product dispersions depend in respect to concentration on the chosen particle

size and shape distributions, their state of dispersion and the intrinsic particle-particle packing characteristics. When transportation over long distances is required, methods to achieve the maximum solids content within the constraints of the final application are strenuously sought.

Figure 1



During fine grinding processes, for example, temperatures of up to 110°C can be reached. This ensures to a large extent the thermal disinfection of the processed mineral. Thus, there occurs generally a significant reduction in the bacterial count, arising initially from contaminated plant and treatment waters and process additives, from that found in the feed material. After such a grinding or high temperature processing step, the dispersions can often be regarded in microbiological terms as of pharmaceutical quality. There are, however, some cells which, due to a certain protection mechanism, are not destroyed by the grinding process but are transferred into a physiologically stressed or dormant state. During slurry product storage, post-dispersion of synthetic products, or during transportation, the temperature can decrease (or rise again) to a level which is favourable to bacterial growth (25-45°C). This growth then proceeds once either infection occurs from outside sources, usually by fluid contamination or even contact with air, or by the further viability of the dormant or stressed cells. It is this post-processing contamination that focuses most attention when considering slurry preservation. Of course, should a processing step involve lower temperature handling or combinations of products then a complete revision of the process environment in respect to bacterial contamination must be made. This is specific to the plant in question and requires extensive analysis of

water systems, settling and waste recovery vessels, and air-borne sources, such as air-conditioning, ventilation etc.

## **2. Organic additives for dispersion stabilisation – a rich nutrient basis**

To be able to produce a suspension having a controlled solids content, often of more than 70 w/w%, special dispersants are required. Without such dispersants, a mixture containing a mineral, such as calcium carbonate, with only 30 w/w% water is no longer flowable. Pigment and filler producers have developed highly active polymer systems, especially for the use of mineral slurries in the paper industry, which allow such concentrations to be achieved without destroying the complex chemistry of a paper machine.

The dispersants, usually based today on salts of polyacrylic acid (PAA), represent a rich supply of organic nutrients for cells. They serve also as both a carbon and energy source. Mineral dispersions, however, also contain a series of other important biologically supportive substrates, which contain oxygen, nitrogen, calcium, magnesium, sodium, potassium, phosphorus, sulfur and iron, all of which are essential for energy metabolism.

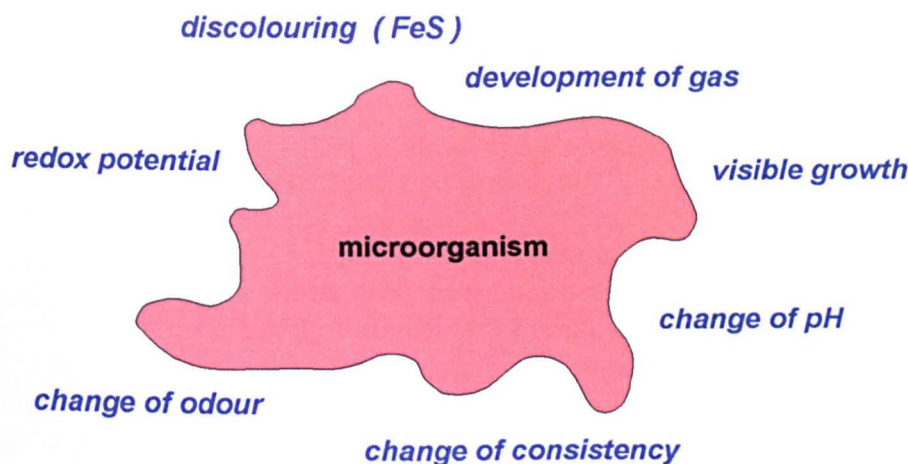
The microbiological colonies, as well as their growth in a mineral dispersion, can be influenced by many different factors, such as:

- Higher solids contents of a dispersion. High solids content makes growth beyond a certain concentration more difficult as the physical space for microorganisms is reduced. As a result, dispersions with high solids contents are often easier to preserve.
- Higher salt concentrations lead to a differential osmotic pressure across the microbial cell walls. The effects are selective to the species present in a system. Usually it is a change in salt concentrations, rather than an absolute level, that can have an inhibiting effect on the growth of microorganisms already extant in the system.

## **3. Microbial contamination and its consequences**

Microorganisms are omnipresent on earth (and maybe beyond) and, of course, mineral dispersions are no exception. Bacterial counts of  $> 10^6$  cfu/ml can lead to unpleasant odour, discoloration, acidification and viscous build-up. Under certain conditions of aeration, followed by stagnation, strong initial aerobic growth, eventually consuming the oxygen present, can subsequently create the conditions for anaerobic growth, which is generally connected with a decrease in the redox potential. Furthermore, the decrease in pH, and the often associated increase in viscosity, can lead to considerable problems for the final user. The rheological properties of mineral dispersions are extremely important for the processing of the product (e.g. pumpability, filtering, rheological flow characteristics in a coating head and in recirculation systems).





Furthermore, there is a risk of uncontrolled deposition (biofilms), which, for example in paper production, could lead to holes and breaks in the paper web.

Clearly, the need for biocide(s) to initiate, preserve and maintain slurry purity is a very important part of the slurry producer's and handler's requirements for efficient application and storage of minerals.

Biocide in the slurry itself, however, is only one aspect of preservation. In order to keep mineral slurries clean in storage tanks and during transportation (truck, railcar, boat) (Photo 1), and to achieve good performance of biocide(s) within the product, the effect of headspace preservation as well as the cleaning of pipes and transportation facilities must not be underestimated (Photo 2). Contamination from headspace can be a major source of biofilm development which has a high potential for the recontamination of mineral slurries (figures 3 and 4). Biofilms can also occur in places where mechanical cleaning is difficult, i.e. dead corners in pipework and storage or reaction vessels. In these cases, where biofilms flourish there is often a limit to the practical chemical disinfection and preservation that can be attained, due to the impermeability of many of these types of films. The need to avoid stagnation in plant design is, therefore, paramount.

Figure 3

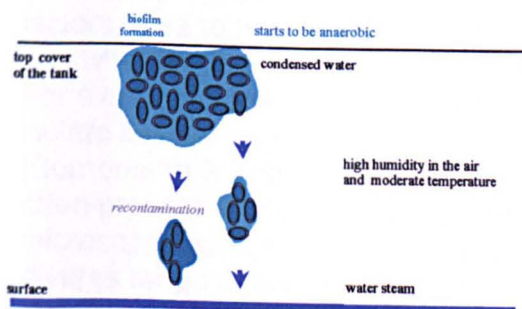
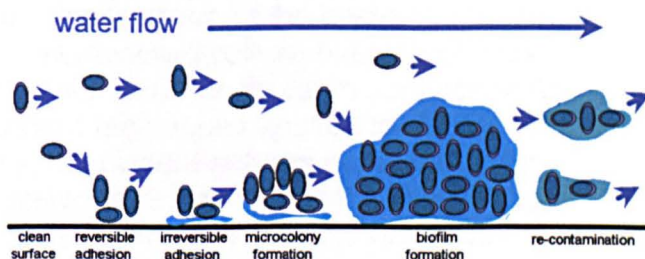


Figure 4





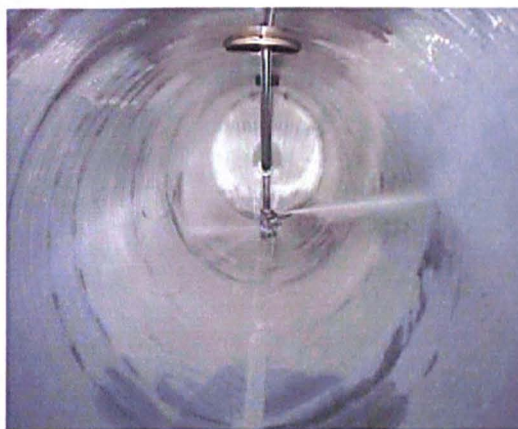
The target, therefore, is not only microbial control within the product itself but also the control within the confines of the immediate product-contacting environment.



Photo 1 : To meet today's logistic requirements, slurry tank vessels with a capacity of up to 16'000 registered tonnes are in operation.

The transport of these large quantities of mineral slurry dispersion makes great demands upon the preservation and brings a new meaning to being "ship-shape".

Photo 2 : Emptied rail tank waggon are cleaned with fresh water under high pressure to guarantee optimum conditions with respect to cleanliness for the next load to be transported.



#### 4. Diversity of bacterial morphologies

The rich nutrient supply, moderate temperatures during transportation and storage of mineral slurry dispersions, as well as a neutral to slightly alkaline pH (7-10), provide an environmental spectrum of conditions for a large number of microorganisms to develop and thrive.

When investigating the microbial diversity that exists in mineral slurries a number of factors have to be considered. These mostly surround questions of how to perform, and what are the effects of, extraction and isolation of the bacteria concerned. For example, it has been demonstrated that standard culture-based techniques only isolate as little as ~ 1 % of the total microbial population found in common soils (Bornemann & Triplett, 1997). Such standard techniques applied to pigment slurries often present similar limitations. Recently, the current authors have shown by microscopic analysis of bacteria concentrated from typical mineral slurries that a diverse range of cell morphologies can be revealed, which by culture techniques remain unidentified (Photo 3 (Schwarzentruber, P., 2001)). Subsequently, the employment of the most modern techniques arising from the field of molecular biology, such as PCR (Polymerase Chain Reaction) amplification of the 16s rDNA, cloning and sequencing and/or RISA (rDNA Internal Spacer Analysis) (Figure 5 and



Photo 4 (Schwarzentruher, P., 2001)), enable the identification of some of the major microorganisms found to occur in mineral dispersions.

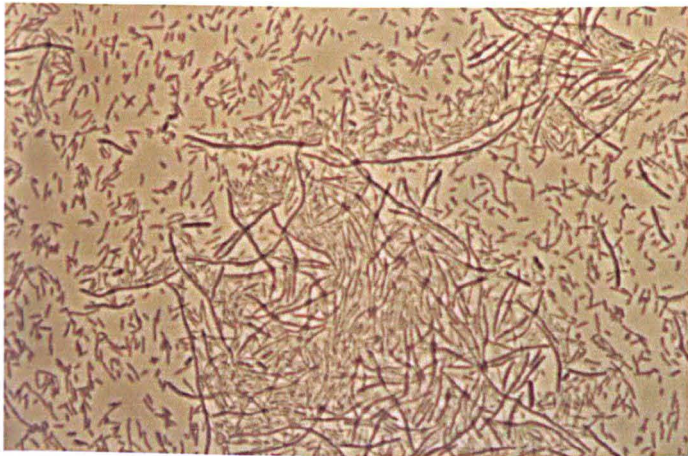


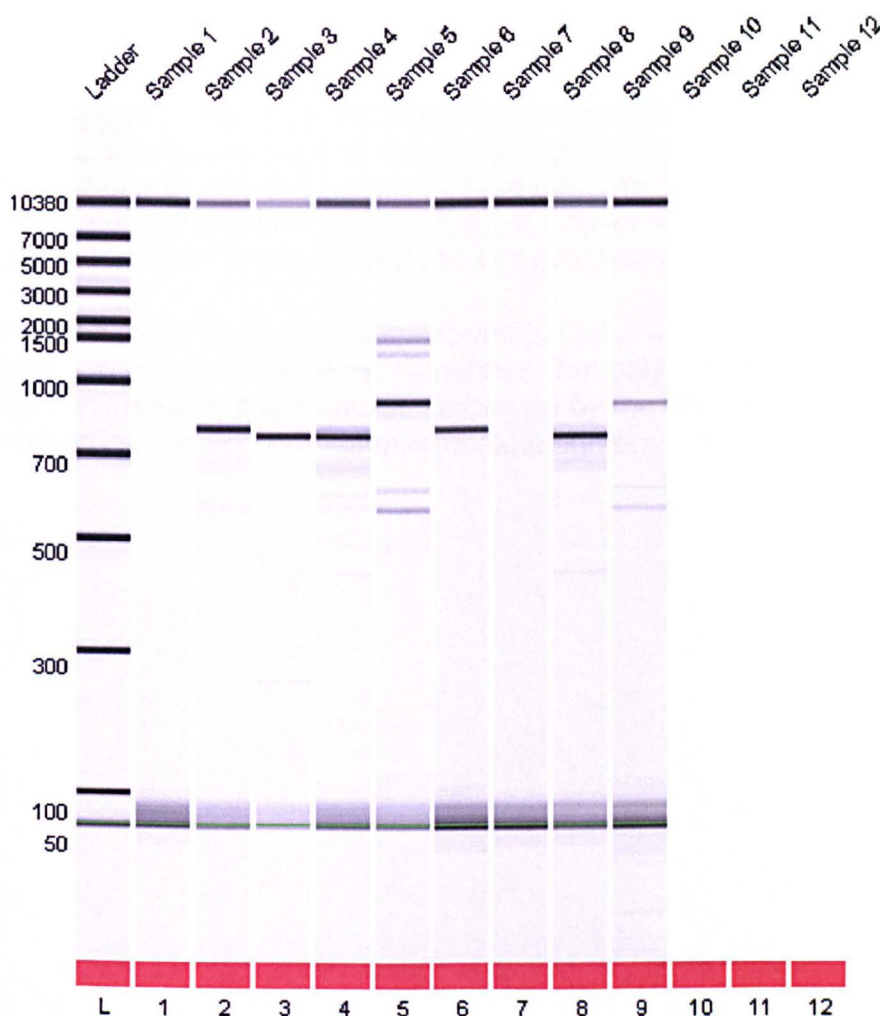
Photo 3:  
Bacterial diversity in a  $\text{CaCO}_3$  slurry sample from France  
100x / light microscopy with phase contrast

Figure 5: rDNA Internal Spacer Analysis (RISA) is a recently developed PCR technique that involves amplifying the spacer region between the 16s and the 23s genes of the rRNA operon.



The length (and sequence) of nucleotides in this region is species specific, and can therefore be exploited to provide an insight into the bio-diversity of a sample. Most importantly, this can be achieved without cloning and sequencing, as a different sized PCR product is produced for each organism present in the slurry.

Photo 4: This gel shows RISA products, prepared by PCR amplification of the 16s-23s spacer region on the rDNA operon of several  $\text{CaCO}_3$  slurry isolates. The number of bands corresponds to the number of rDNA operons inherent to each species while the size and sequence of each spacer region is species specific.



Depending on the nutrient supply, some species can be favoured. Similarly, in certain cases often found in mineral slurries, Methanotrophs and Methylotrophs can be favoured not only due to original nutrient sources but also in respect to the biocides used. A wide variety of bacteria are known that can grow on methanol, methylamine, or formate, and, in the case of Methanotrophs, also on methane. If, therefore, pure formaldehyde donors are used as biocide it cannot be ruled out that bacteria of these genera will accumulate over a certain period of time. This is just one example of how the spectrum of bacteria in relation to nutrient before and during preservation must be considered and not just the original dominant contamination.

The genera most frequently occurring in mineral dispersions, however, are the pseudomonads. These straight or curved gram-negative rods have very simple nutritional requirements and grow chemo-organotrophically at neutral pH and at temperatures in the mesophilic range. One of the striking properties of many species of pseudomonads is the wide variety of organic compounds that are used as carbon

and energy sources. Some species of these genera also show a tendency to biofilm formation.

The gram-positive *Micrococcus* and *Staphylococcus* are both aerobic organisms with a typical respiratory metabolism. Gram-positive cocci are relatively resistant to reduced water content and have the potential to tolerate drying and high salt levels fairly well. Gram-positive cocci can most readily become introduced into a mineral dispersion via the addition of dispersants (salts of polyacrylic acid (PAA)). Usually, their growth is suppressed by the gram-negative bacteria that are dominant in the matrix, but this naturally depends on the stage of dispersant addition in the process and the prior existence or otherwise of dominant organisms.

An overview of the major microorganisms found to occur in mineral dispersions is shown in table 1, although it has to be mentioned that only those species which could be repeatedly confirmed by the methods employed by the current authors have been listed, and, therefore, the list is by no means exhaustive.



Table 1

Group	Genus	Species
Aerobic / microaerophilic, motile, helical / vibrioid gram-negative bacteria	<i>Bdellovibrio</i>	<i>B. bacteriovorus</i>
Gram-negative aerobic / microaerophilic rods and cocci	<i>Acidovorax</i>	<i>A. delafieldii</i>
	<i>Agrobacterium</i>	<i>A. radiobacter</i>
	<i>Alcaligenes</i>	<i>A. xylosoxidans</i>
	<i>Flavobacterium</i>	<i>F. indologenes</i>
	<i>Methylobacterium</i>	<i>M. extorquens</i> <i>M. mesophilicum</i>
	<i>Pseudomonas</i>	<i>P. aeruginosa</i> <i>P. alcaligenes</i> <i>P. cepacia</i> <i>P. diminuta</i> <i>P. fluorescens</i> <i>P. luteola</i> <i>P. maltophilia</i> <i>P. mendocina</i> <i>P. paucimobilis</i> <i>P. pickettii</i> <i>P. pseudoalcaligenes konjaci</i> <i>P. putida</i> <i>P. stutzeri</i> <i>P. testosteroni</i> <i>P. vesicularis</i>
	<i>Rhizobium</i>	<i>R. spp.</i>
	<i>Sphingobacterium</i>	<i>S. spiritivorum</i>
	<i>Aeromonas</i>	<i>A. caviae</i> <i>A. hydrophilia</i> <i>A. salmonicida achromogenes</i> <i>A. salmonicida masoucida</i>
	<i>Chromobacterium</i>	<i>C. spp.</i>
Facultatively anaerobic gram-negative rods	<i>Vibrio</i>	<i>V. metschnikovii</i> <i>V. parahaemolyticus</i>
	<i>Hyphomicrobium</i>	<i>H. vulgare</i>
Budding and / or appendaged bacteria	<i>Leptothrix</i>	<i>L. discophora</i>
Sheathed bacteria	<i>Micrococcus</i>	<i>M. luteus</i> <i>M. roseus</i> <i>M. varians</i>
Gram-positive cocci	<i>Staphylococcus</i>	<i>S. capitis</i> <i>S. cohnii cohnii</i> <i>S. lentus</i> <i>S. sciuri</i> <i>S. xylosus</i>
	<i>Agromyces</i>	<i>A. ramosus</i>
Irregular, non-sporing gram-positive rods		

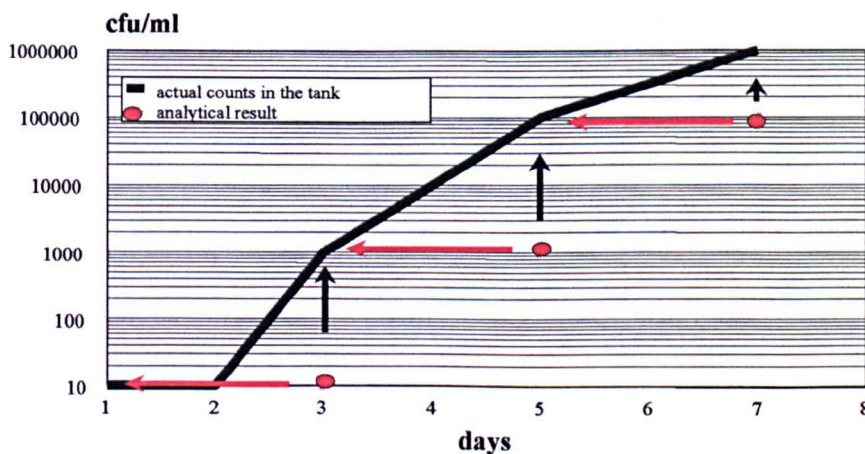
## 5. Prevention and control of microbial activity – real time monitoring and evaluation

Prevention of contamination caused by microorganisms and the effects arising therefrom have economic consequences. These consequences must not be underestimated. For example, significant changes in the rheology of mineral dispersions can lead to a standstill in paper production. Similarly, continual false application of biocide(s) arising from an over-reaction philosophy can have equally dramatic economical effect.

To achieve the goal of optimal control of microbial contamination continuous monitoring of the biological activity in a system and well-balanced housekeeping (i.e. storage tank and transport cleanliness) are necessary. Together with monitoring, the process of optimal dosing and selection of biocide(s) becomes an integrated part of the strategy. It is therefore necessary to consider carefully the options available both currently and those under development for isolation and monitoring of the bacteria, as rapid identification and colony determination are crucial to the benefits that can be derived from optimising the control strategy.

Over the last years, classic methods for the determination of the total viable count have become established in the white mineral industry. The use of agar substrates in a Petri dish, or on a plastic film, are primarily suitable for a first count (and isolation) of aerobic or facultatively anaerobic microorganisms. This method can also be applied for not too extreme anaerobes provided the plates are incubated in an anaerobe jar. A significant disadvantage of this plate method for determining the total viable count is the long incubation time (culture enrichment) required (typically 48-72 hours) which strongly impairs the ability to apply quality control, and hinders online production security. Figure 6 clearly shows that, due to the long incubation times of traditional methods, the result does not reflect the current situation in the storage tank but the situation as it was some ~ 48 hours before.

Figure 6



New applications of established instrumental techniques, such as electronic cell counting, based on a Coulter-counter, as well as methods for vital counting (vital staining), offer interesting alternatives to the agar substrates. These options are



reviewed briefly in the next section. Furthermore, we go on to consider novel methods of electro-optically identifying the bacteria undergoing vital staining.

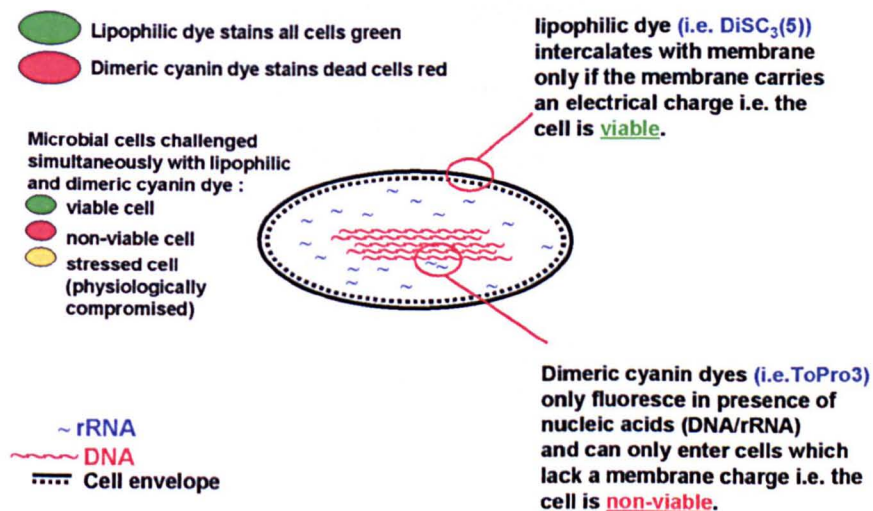
### *Electronic cell counting: the Coulter-counter*

The cells to be counted are suspended in a conductive aqueous solution, an electrolyte. An exactly determined small volume of this suspension is made to flow through a narrow capillary orifice which connects two chambers filled with the electrolyte. In each of these two chambers an electrode is immersed into the liquid. A current is flowed between the two electrodes, and the electrical resistance, which is generated by the narrow orifice, is measured. A biological cell passing the orifice leads to a temporary increase in the resistance as the electrical conductivity of the cell is much lower than that of the electrolyte. The voltage pulse generated by the increase in the resistance is amplified and electronically registered. In this way, the number of cells passing the orifice is obtained. Although this procedure enables the determination of the number of cells, it does not provide any information about the physiological state of the microorganisms. For this purpose, the cell suspension has to be transferred into a liquid culture medium and incubated.

### *Vital staining*

In the case of ecological investigations, the customary methods for the determination of the number of cells do not show whether the detected microorganisms are physiologically active in the place where they are naturally occurring, only that they are active under the specific culture conditions employed. There is no doubt that the number of microorganisms with active metabolism in a mineral dispersion is considerably higher than the number found by the standard methods for the determination of the total viable count. On the other hand, it is often found that, in their natural habitat, a considerable number of the counted cells are in a state of rest and are physiologically inactive. For this reason, a series of staining methods have been developed by which it would be possible to recognise viable, metabolically active, microorganisms directly under the microscope, and to distinguish them from dead or inactive cells (Figure 7) (Fry, 1990; Hall *et al.*, 1990; Lloyd & Hayes, 1995). Fluorescent dyes are preferably used for vital staining, as fluorescent cells are considerably easier to recognise and count than non-fluorescent ones.

Figure 7





## Novel real-time monitoring and evaluation

To be able to react increasingly effectively to microbial contamination in the production of millions of tonnes of mineral slurries per year, and to optimise the biocide consumption costs, a method which measures directly the total viable count in real-time is urgently required. Such a method is nearing completion in its development.

A combination of the Coulter-counter and fluorescence techniques has been investigated and developed in application to mineral slurries by the current authors and is now seen to offer a potential solution.

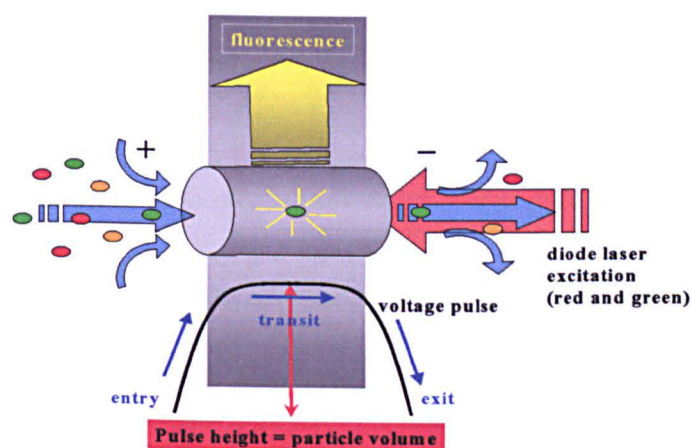


Figure 8:

Schematic view of the principle of CellFacts II®. Particles (or cells) in an electrolyte are sucked through a measuring orifice having a diameter of 30  $\mu\text{m}$ . The laser (red or green) is focused at the measuring orifice.

With such a method the number of particles is counted and the volume determined in parallel. By staining the cells and determining the emitted fluorescence, the physiological state of the individual cells can be determined, so culture enrichment is no longer necessary. Without the need for enrichment, the result is given in real-time. This methodology is just emerging in practical field trials for mineral slurry contamination control.

## 6. Constraints on suitable types of microbiocides

Today, biocides for the preservation of mineral slurries have to meet far more requirements than showing demonstrable bactericidal action alone. Some of the most important criteria which decide whether a bactericidal agent can be used for the preservation of mineral slurries can be summarised as follows:

- Must be thermally stable up to a minimum of 60°C.
- Needs to have a positive redox potential.
- Its inhibition of nitrification must be < 30 % (in dilution) in slurry so as not to impair the nitrification in subsequent waste water purification plant.
- It must be biodegradable to a level greater than 80 w/w%, preferably 100 w/w% (OECD 301D).

- May not generate a negative inhibition area around finished paper or application surface - this applies to the need to prevent extraction of biocide from paper and packaging materials when in contact with foodstuff.
- Must have no negative influence on other mineral dispersion properties.
- Required to have Regulatory Approval (e.g. FDA, BgVV, Nordic Ecolabelling).

Among a large number of bactericidal agents the following ones have proved to be successful as preservatives in mineral dispersions. Once again, this list should not be considered as exhaustive: many biocides which have proven efficacy have either fallen out of favour due to regulatory requirements or even environmentally-related perceptions, and many biocides remain to be discovered or, if already existing, to be applied in this field. We go on to consider some currently used biocides and identify a newly applied biocide in this field.

### *2-Bromo-2-nitro-propan-1,3-diol (Bronopol)*

Bronopol has a broad spectrum of antibacterial activity and belongs to the group of aldehyde-releasers as well as to the group of activated halogen-compounds. It is widely used as a preservative of pharmaceutical and cosmetic products (Croshaw *et al.*, 1964; Storrs & Bell, 1983). Another important emerging application is the use as a preservative in the filler and pigment industry (e.g. calcium carbonate slurries).

A considerable disadvantage, however, is that the compound is not heat-stable. At a temperature of 60°C under alkaline conditions the active agent is completely decomposed within a few hours.

### *Isothiazolin (MIT / CIT / BIT)*

Isothiazolin biocides, such as 1,2-benzisothiazolin-3-one (BIT), 2-methyl-4-isothiazolin-3-one (MIT) and 5-chloro-2-methyl-4-isothiazolin-3-one (CIT) are widely used as environmental biocides as well as preservatives for filler- and pigment-systems.

Isothiazolin has a high potential for sensitising (Weaver *et al.*, 1985) and compounds based on isothiazolin are not heat-stable (Willingham & Mattox, 1990). They are today mainly used in combination with bronopol or ethylene glycol hemiformal for preservation in the pigment and filler industry.

### *Phenol derivatives (e.g. o-Phenylphenol)*

Phenol, also termed carbolic acid, and the phenol derivatives could also be considered as acids because of their acidity and the resulting capacity to form stable salts. Carbolic acid and soaps have been widely used as cleansers and disinfectants including in the home and in medical environments. Today a wide range of phenols are used for the protection of materials. Since o-phenylphenol is used to preserve citrus fruits because it has the most favourable toxicity data (Paulus, 1993) this phenol derivative especially has rapidly gained importance as a preservative for the protection of pigment slurries, and this is new. It is, however, not ideal as a

disinfectant in slurries due to the relatively low solubility level, although its value in preservation is being established; its application forms in fact being under current patent rights.

#### *Aldehydes (e.g. glutaraldehyde)*

Aldehydes have a broad spectrum of activity. Bacteria, fungi, spores and viruses are killed or inactivated. This, however, depends on the effective or differing exposure times.

The pressure from the pigment and filler industry to abstain from using formaldehydes is increasing. Considering that 200 ppm per tonne pigment/filler have to be used for a sufficient preservative action, it is clear that in the production of paper (wet end), quantities of formaldehyde can become released. Due to the high vapour pressure of formaldehyde, vapour is frequently released into the air during and after the disinfection or preservation procedure and concentration levels can build in enclosed production environments. Furthermore, this active agent has been classified as sensitising.

#### *Formaldehyde-releasing compounds (e.g. ethyleneglycol-hemiformals)*

Formaldehyde, as such, is often too volatile and too reactive to be used as a microbiocide for the protection of white minerals. It additionally produces unwelcome side-effects such as an increase in viscosity, and has an insufficiently balanced range of activity. One, therefore, continually searches for formaldehyde-releasing compounds which do not exhibit the disadvantageous formaldehyde effects but maintain or even improve the antimicrobial action of formaldehyde. However, the environmental limitations on released formaldehyde still apply.

Formaldehyde-releasing compounds can be found as both solids and liquids, water soluble or oil soluble, alkaline, neutral, or slightly acidic. According to their composition, the ethylene glycol-hemiformals are especially effective against bacteria and therefore useful for the so-called "in-can", or storage container, protection of a large variety of industrial fluids, mainly together with other active ingredients, e.g. fungicides. Also, the protection of white mineral slurries using ethylene glycol-hemiformal has proven to be effective.

#### *3,5-Dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione (DAZOMET)*

3,5-Dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione has a broad spectrum of high activity which covers bacteria, fungi and yeast, indicating that the substance is a specialised formaldehyde-releasing compound.

The spectrum of activity is attractive for application in a number of industrial systems, e.g. as a broad spectrum microbiocide which prevents fungal blooms in metal working fluid systems. However, there are limitations, including the finding that 3,5-Dimethyl-tetrahydro-1,3,5-2H-thiadiazine-2-thione is very unstable at increased temperatures. Under alkaline conditions it is found that more decomposition products are formed than under pH neutral conditions (e.g. wet-end system).

The intermediate breakdown products formed under alkaline conditions have a strong smell. The preservation of filler and pigment slurries with thione has a high risk for bad smelling slurries and end product, such as paper, especially when heated in a humid environment.

### *2,2-Dibromo-3-nitrilopropionamide (DBNPA)*

DBNPA has a broad spectrum of high activity with a low environmental impact which covers bacteria, fungi and yeast.

These antimicrobials are fast acting, but are temperature sensitive and will decompose exothermally (liberating heat) at elevated temperatures. They are today mainly used in combination with other biocides, such as bronopol or ethylene glycol hemiformal for preservation in the pigment and filler industry.

### *Methylene bithiocyanate (MBT)*

MBT is an effective microbiocide for bacteria, algae, yeast and fungi, but has a limited stability. The acidic microbiocide must preferably be added at a point of good agitation to a storage tank with adequate turnover.

## **7. Regulatory, safety and environmental issues - some practical points**

Since mineral dispersions might be used in the production of food packaging the substances used have usually to comply with most of the relevant laws, such as BgVV Germany, FDA USA and current and emerging EU directives.

With respect to the relevant laws in different countries not only the quality of the biocides but also possible limitations of the quantity have to be considered. Special attention has to be paid to the fact that different countries may also impose different limitations of the quantity. Below, we identify some of the most relevant criteria applicable today.

### *Biocidal Product Directive 98/8/EG*

The aim of the Biocidal Product Directive 98/8/EG, passed in February 1998, is to harmonise the marketing of biocidal products (this includes products such as wood preservatives, disinfectants, pesticides and antifouling paints) within the EU. This results in comprehensive new regulations being laid down so that the supply of active substances will continue to be ever more severely restricted.



## *Risk for humans and environment*

The requirements with respect to the toxicity of an antimicrobial substance primarily depend on the intended use. The most rigid requirements have to be met by products used for food preservation as people are exposed to high concentrations of them (0.05 – 0.50 %) over a long period of time, often daily. For this reason it is most important that these substances are recognised as safe for health.

With respect to the intended use it is necessary to consider not only the quality of the biocide but also possible limitations of the quantity especially with respect to biodegradability, air pollution, by formaldehyde for example, and the risk of skin-sensitisation for humans. To review this area of legislation is beyond the scope of this article.

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# CellFacts II – Single Cell Analysis in Real Time

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**Summary:** CellFacts II integrates electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics of individual cells in a conductive fluid. The instrument has been optimised to detect and enumerate viable and non-viable cells in fluid samples with varied particulate content, i.e. total viable counts, with discrimination of the physiological status of the individual cells.

The study shows the analysis of the physiological state of individual cells in a population, effectively in real-time, enabling the rapid determination of the effect of antimicrobial agents on these cells i.e. rapid determination and optimisation of antimicrobial agents in aqueous paint systems.

**Keywords:** CellFacts; fluorescence; impedance; physiological status; total viable count

## Introduction

Traditional microbiological analyses for determination of the presence of low levels of contaminating microorganisms, such as Plate Count, Petrifilm or Easicult, are lengthy, often taking from 2 to 3 days to complete. In addition, there are various other factors to be considered, such as, for example, type of culture medium, partial pressure of oxygen (aerobic / anaerobic), selectivity, pH value and many more.<sup>[1-2]</sup>

A rapid method that could provide an accurate assessment of the number of microorganisms present would allow positive release of the paint product in the knowledge that it was free from effective contamination.

CellFacts II integrates electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics (e.g. viability, physiological status, speciation) of individual particles or cells in a conductive fluid. The instrument has been optimised to detect and enumerate viable and non-viable cells in fluid samples with varied particulate content, i.e. total viable counts,

with discrimination of the physiological status of the individual cells (also discrimination between cells of different sizes, e.g. between yeast and bacteria).

The following study shows the analysis of the physiological state of individual cells in a population, effectively in real-time, enabling the rapid determination of the effect of antimicrobial agents on these cells, i.e. rapid determination and optimisation of antimicrobial agents in aqueous paint systems. Since antimicrobial agents have to meet the new Biocidal Product Directive (BPD), and paint systems will be valued according to the directive RAL UZ12a (Blauer Engel) this level of proactive microbiological control becomes even more important.

### The Operating Principle

CellFacts II uses patented technology integrating electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics. This is used to determine the viability, physiological status and speciation of individual particles or cells in a conductive fluid (Figure 1).

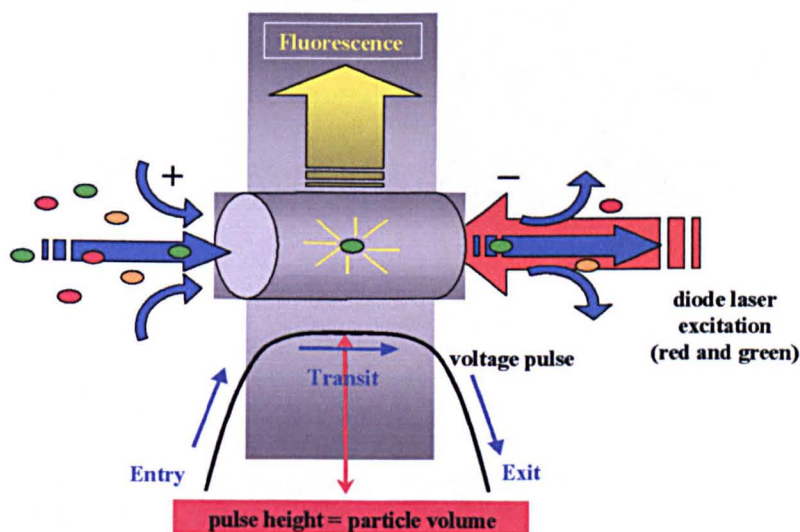


Figure 1. Schematic presentation of the CellFacts II principle. Particles (or cells), in a conductive fluid, pass through a 30  $\mu\text{m}$  diameter orifice which has been laser etched in a 80  $\mu\text{m}$  sapphire disk.

Two diode lasers (red and green) are focussed on the orifice. As a particle (or cell) enters the orifice it displaces its own volume of electrolyte and this generates a voltage pulse, the magnitude of which is directly proportional to the volume of the particle. This signal is analysed in

considerable detail via the software and generates information on particle (or cell) size and the numbers within a given population. It may also be used to trigger fluorescence characterisation of the particle as it transits the orifice, i.e. the integration of impedance and fluorescence data. A key feature is the orifice block detection systems which ensure the instrument operates irrespective of the size heterogeneity of the particles on the sample side of the orifice and results in an extremely effective anti-blocking system.<sup>[3]</sup>

The instrument has a modular configuration (Figure 2) to meet the requirements of the broad spectrum of applicable market sectors.

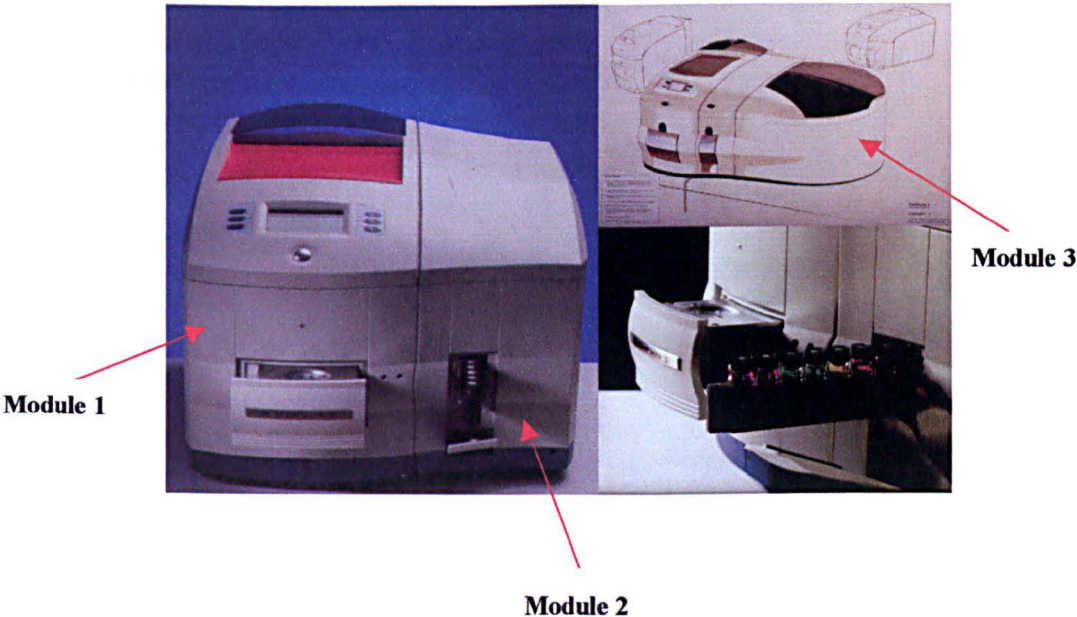


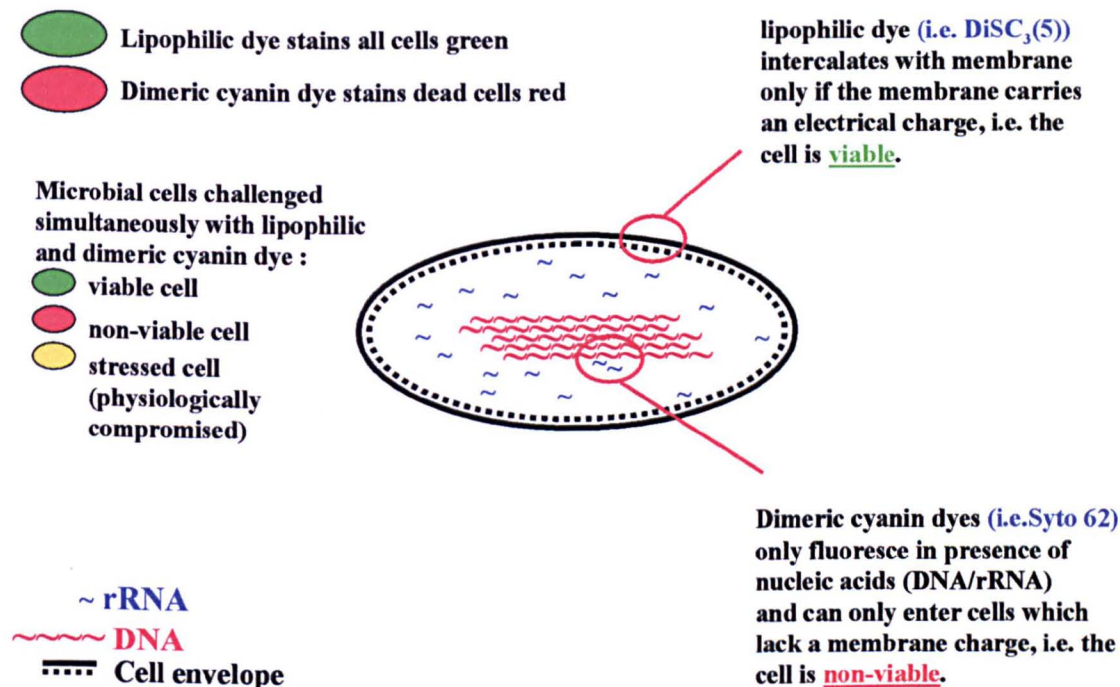
Figure 2. CellFacts II Modules:

- Module 1*      Analysis module, integrating both impedance and fluorescence measurements of individual particles or cells.
- Module 2*      Reagent addition module. This unit facilitates the addition of up to seven different reagents enabling multiple, complex, automated analyses to be undertaken for each sample.
- Module 3*      Shown here in the original concept drawing, is an automated sample-handling unit with sample mixing and bar code facilities.



## Fluorescence: Characterising the Physiological Status of Cells

By following a protocol of minimal staining with dimeric cyanine nucleic acid dyes and lipophilic cationic dyes (membrane potential dyes, i.e. viability indication dyes) it is possible to characterise the physiological status of individual cells (Figure 3).<sup>[4-5]</sup>



## Results

A study was carried out comparing the CellFacts II method, using a patented sample preparation technique which allows high solids suspensions to be analysed by removing preferentially microbial material, with traditional techniques, such as Plate Count, for evaluating the potential application in aqueous paint systems. The preservative properties of commercially available paint systems were also tested and different antimicrobial agents were compared against one another. Since antimicrobial agents have to meet the new Biocidal Product Directive (BPD), and paint systems will be valued according to the directive RAL UZ12a (Blauer Engel) this proactive evaluation methodology will become even more important.

## Total Viable Count

On comparing the total viable count of all the samples tested (Table 1) it becomes clear that the results using the new technology CellFacts II tend to be somewhat higher than those of the traditional plate count method. This result was in fact expected, and can be explained due to the specific test conditions chosen for the traditional culture evaluation (nutrient, pH, temperature). This shows that not all cells are detected by the plate count method ! It is possible that cells are present in a system which can indeed have a negative impact on the properties of the product but that traditional methods cannot detect them. These cells could be successfully detected by the CellFacts II method, and, what is new, in real time.

Table 1. Total Viable Count of 16 Aqueous Paint Systems

Sample	PCA <sup>a)</sup> (cfu/ml) <sup>b)</sup>	CellFacts II (cfu/ml)
Interior Emulsion Paint 1A	< 100	< 100
Interior Emulsion Paint 1B	< 100	< 100
Exterior Emulsion Paint 1C	< 100	< 100
Exterior Emulsion Paint 1D	< 100	< 100
Interior Emulsion Paint 2	< 100	$3.0 \times 10^2$
Exterior Emulsion Paint 2	< 100	< 100
Interior Emulsion Paint 3	$2.0 \times 10^2$	$1.3 \times 10^3$
Exterior Emulsion Paint 3	< 100	< 100
Exterior Emulsion Paint 4A	$1.0 \times 10^2$	$7.8 \times 10^3$
Interior Emulsion Paint 4B	< 100	$3.5 \times 10^3$
Exterior Emulsion Paint 4C	< 100	< 100
Interior Emulsion Paint 4D	< 100	< 100
Interior Emulsion Paint 5A (without biocide)	< 100	$1.8 \times 10^3$
Exterior Emulsion Paint 5B (without biocide)	$7.0 \times 10^2$	$6.7 \times 10^3$
Interior Emulsion Paint 5C (without biocide)	$5.0 \times 10^4$	$9.4 \times 10^4$
Exterior Emulsion Paint 5D (without biocide)	$8.0 \times 10^4$	$4.9 \times 10^5$

a) PCA = Plate Count Agar

b) cfu = cell forming unit

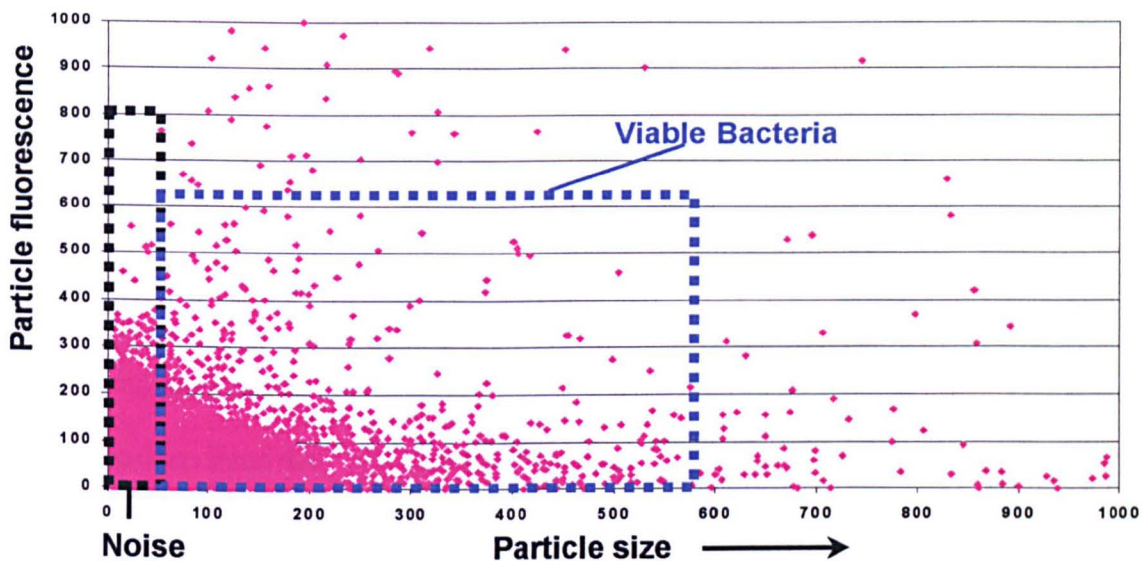


Figure 4. This graph clearly shows the area of the viable bacteria defined by fluorescence and particle size. The total viable count detected in the sample shown (Interior Emulsion Paint 4B) was  $3.5 \times 10^3$  cfu/ml.

### Stability of Preservation

The samples in the series 1 - 4 were tested for their existing preservative properties. For this purpose, the samples were inoculated with a quantity of  $5.0 \times 10^4$  bacteria per ml isolated from aqueous paint systems. After the subsequent storage of the samples for 24 hours at  $30^\circ\text{C}$ , the total viable count was determined by means of CellFacts II. Additional inoculations were made until the total viable count of a sample was  $> 10^4$  cfu/ml (Table 2). The number of possible additional inoculations without viable contamination gives an indication of the quality of the preservative action in the product. The results show that significant differences can be found in the samples analysed. In this study the paints from series 1 could be identified as having the best preservative properties.



Table 2. Stability of Preservation of 12 Aqueous Paint Systems

Sample	TVC <sup>a)</sup> before Inoculation	Number of Inoculations
Interior Emulsion Paint 1A	< 100	4
Interior Emulsion Paint 1B	< 100	4
Exterior Emulsion Paint 1C	< 100	3
Exterior Emulsion Paint 1D	< 100	4
Interior Emulsion Paint 2	$3.0 \times 10^2$	1
Exterior Emulsion Paint 2	< 100	2
Interior Emulsion Paint 3	$1.3 \times 10^3$	1
Exterior Emulsion Paint 3	< 100	2
Exterior Emulsion Paint 4A	$7.8 \times 10^3$	1
Interior Emulsion Paint 4B	$3.5 \times 10^3$	1
Exterior Emulsion Paint 4C	< 100	3
Interior Emulsion Paint 4D	< 100	2

a) TVC = total viable count

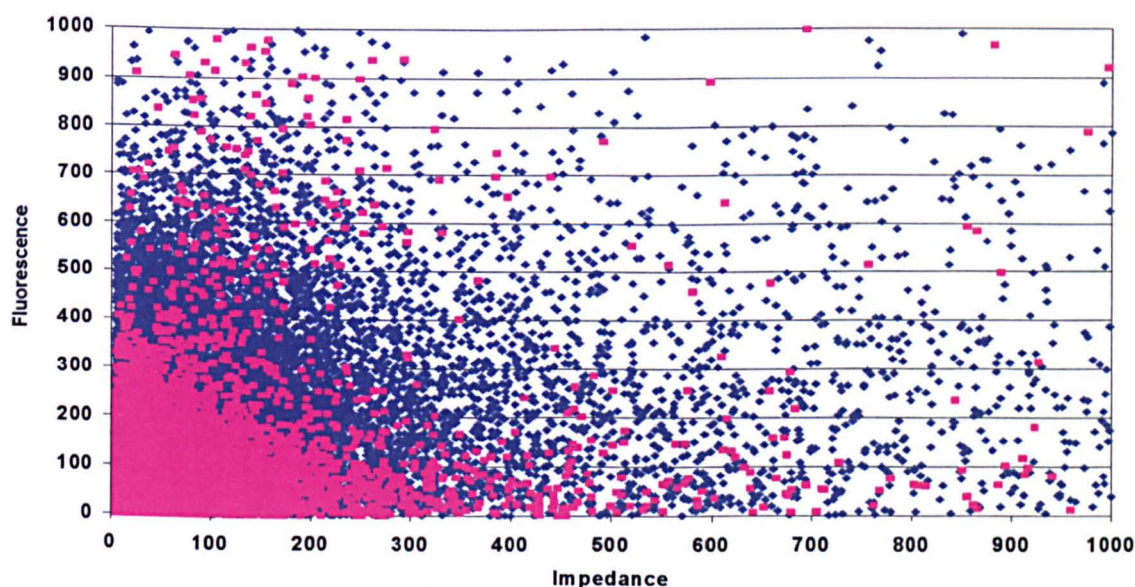


Figure 5. This graph shows the Interior Emulsion Paint, taken from series 3, before (violet) and after (blue) the inoculation amounting to approx.  $5.0 \times 10^4$  cells/ml. The initial total viable count measured was  $1.3 \times 10^3$  cfu/ml. After the inoculation and incubation for 24 hours at 30°C, however, the total viable count was  $2.6 \times 10^6$  cfu/ml. The residue of the biocide in the sample was not sufficient to prevent a further contamination of viable microbial activity.

## Performance of Different Antimicrobial Agents in Aqueous Paint Systems

In a further test the bactericidal properties of different active agents were compared by means of CellFacts II. The following substances were used in this study:

- 2-methyl-2H-isothiazole-3-one (MIT) / 5-chloro-2-methyl-2H-isothiazoline-3-one (CMIT) (CMIT:MIT = 3:1)
- 1,2-benzisothiazole-3(2H)-one (BIT)
- o-phenylphenol (OPP)
- 2-bromo-2-nitro-propane-1,3-diol (bronopol)

For this purpose, sterile aqueous paint samples were mixed with controlled quantities of the active agents, which are usually added in production, and stored for 24 hours at 30°C to achieve thorough mixing of the active agent. The samples were then inoculated with bacteria isolated from aqueous paint samples. The measurements with CellFacts II were made after 15 min., 30 min., 60 min., 2 hours, 4 hours, 6 hours and 8 hours. Both the decrease of the viable bacteria as well as the increase of the dead bacteria were measured.

Table 3. Performance of Different Antimicrobial Agents in Aqueous Paint Systems

Biocide	Time	% of cells detected as viable	% of cells detected as dead
MIT/CMIT	0 min	100	0
MIT/CMIT	15 min	97	3
MIT/CMIT	30 min	72	28
MIT/CMIT	60 min	16	84
MIT/CMIT	2 h	0	100
BIT	0 min	100	0
BIT	15 min	100	0
BIT	30 min	87	13
BIT	60 min	69	31
BIT	2 h	47	53
BIT	4 h	38	62
BIT	6 h	35	65
BIT	8 h	32	68
BIT <sup>a)</sup>	24 h	32	68

a) To detect a possible deficiency in the action of the biocide, the samples were again measured 24 hours later.



OPP	0 min	100	0
OPP	15 min	37	63
OPP	30 min	0	100
Bronopol	0 min	100	0
Bronopol	15 min	98	2
Bronopol	30 min	82	18
Bronopol	60 min	65	35
Bronopol	2 h	30	70
Bronopol	4 h	9	91
Bronopol	6 h	0	100

Table 3 shows that BIT has a deficiency in its action against some of the bacteria used in this study, which is not necessarily recognised by the traditional methods due to their culture selectivity. Furthermore, it could be shown that the fastest bactericidal action is achieved with OPP, whereas with Bronopol the bactericidal effect only started after 6 hours.

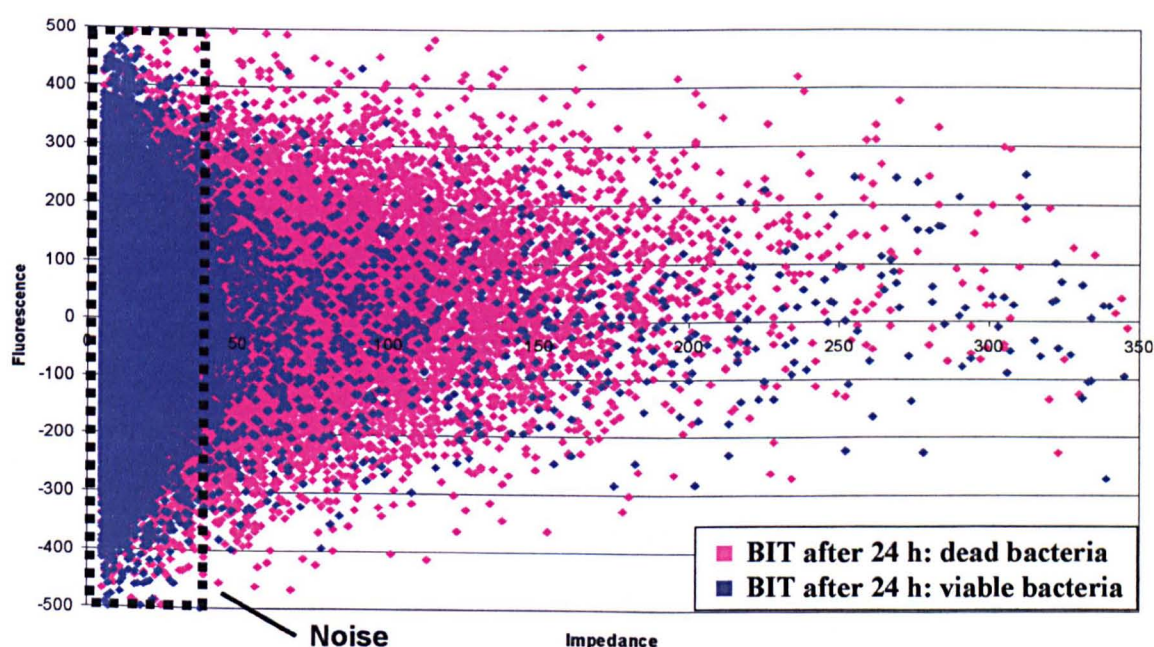


Figure 6. In this graph the deficiency in the action of BIT is illustrated. After 24 hours reaction time there are still viable bacteria. BIT is well-known for its deficiency in the action against some species of *Pseudomonas*<sup>[6]</sup>.

## Conclusion

CellFacts II has been shown to have a highly application-oriented functionality for both analytical flexibility and minimal operator intervention. The sensitivity and specificity of each particular analysis is given by the ability to determine the particle heterogeneity of the sample and the staining characteristics of the microbial population, which in turn is determined by the physiological status of the individual cells. Also discrimination between cells of different sizes, e.g. between yeast and bacteria, is possible.

The analysis of the physiological state of individual cells in a population, effectively in real-time, enables the rapid determination of the effect of antimicrobial agents on these cells, i.e. rapid determination and optimisation of antimicrobial agents.

[1] Hall, G.H., Jones, J.G., Pickup, R.W., Simon, B.M., 1990. Methods to Study the Bacterial Ecology of Freshwater Environments. In Grigorova, R., Norris, J.R. (eds.), Methods in Microbiology, 22, pp. 181-209. London – San Diego – New York: Academic Press.

[2] Fry, J.C., 1990. Direct Methods and Biomass Estimation. In Grigorova, R., Norris, J.R. (eds.), Methods in Microbiology, 22, pp. 41-85. London – San Diego – New York: Academic Press.

[3] Schwarzenhuber, P., Gane, P.A.C., 2002. Application of Microbiocides for the Storage Protection of Mineral Dispersions. In Paulus, W., Directory of Microbiocides for the Protection of Materials and Processes. Dordrecht: Kluwer Academic Publishers.

[4] Lloyd, D., Hayes, A.J., 1995. Vigour, Vitality and Viability of Microorganisms. FEMS Microbiol. Lett., **133**, pp. 1-7.

[5] Schwarzenhuber, P., 2001. Microbiological Characterisation of CaCO<sub>3</sub> Slurries; Interim Report presented at the University of Warwick, Coventry, UK.

[6] Paulus, W., 1993. Microbiocides for the Protection of Materials – A Handbook. London: Chapman & Hall.

# **CellFacts II - Echtzeit-Analyse einzelner Zellen**

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CellFacts II eröffnet neue Möglichkeiten in der mikrobiologischen Analytik. Ausgehend vom Prinzip der elektrischen Impedanz, welches bereits seit 5 Jahren in der CellFacts I-Technologie praktisch umgesetzt wird, addierte man im CellFacts II-Analyser ein zeitgleiches Fluoreszenzsignal, wodurch die Bestimmung der Anzahl, Grösse und physiologische Charakteren von mikrobiologischen Zellen in wässrigen Medien in Echtzeit möglich ist. Die Vorteile gegenüber einem Flow-Cytometer oder traditionellen Plate Count-Methoden liegen klar auf der Hand. Doch was kann die neue CellFacts-Technologie für die Farben- & Lackindustrie leisten ?

## **Traditionelle Techniken**

Die mikrobiologische Analyse von Flüssigkeiten steht im direkten Zusammenhang mit folgenden Parametern: Temperatur, pH, Nährstoffangebot und -konzentration, Chemischen Reaktionen/Kreuzreaktionen von Produkten, Sauerstoff-/Kohlendioxidgehalt, Kultur-Fluoreszenz (Autofluoreszenz), Redox-Potential, Biomasse u.a.

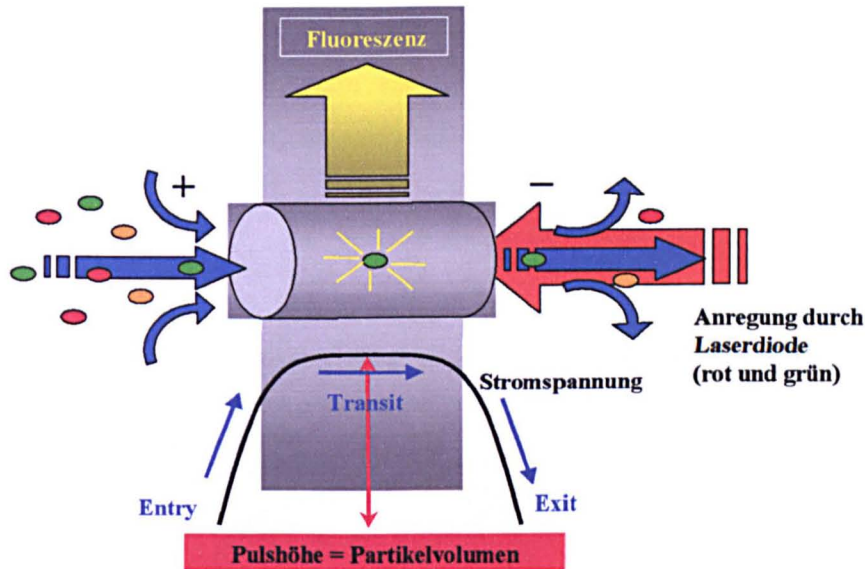
Diese Parameter haben Einfluss auf zelluläre Aktivitäten z.B. der Enzyme, der DNA bzw. der RNA, welche die primären Variablen der 'Performance' einer mikrobiologischen Flora oder zellulärer Kultur sind.

Lange Inkubationszeiten sowie signifikante Selektion von Mikroorganismen (die Inhaltsstoffe von Nährmedien müssen auf die zu kultivierenden Keime, d.h. deren biochemische Stoffwechselcharakteristika, abgestimmt sein) sind bei traditionellen Methoden unbefriedigend.

Weitere Probleme bei der Flow-Cytometrie zur Teilchenzählung oder zum Nachweis von Keimen sind die unzureichende Darstellung der Morphologie und des physiologischen Status der Zellen sowie hohe Nachweisgrenzen. Auch sind hier echte On-Line-Anwendungen nicht möglich, da komplexe Reagenzdosierungen durchgeführt werden müssen.

## Das Funktionsprinzip

Die Integration der Elektrischen Impedanz mit zwei rauscharmen Lasern, im roten Bereich bei 635 nm (zur Umgehung der Autofluoreszenz) und im grünen Bereich bei 540 nm, ermöglichen die Bestimmung der Anzahl und der Grösse mikrobiologischer Zellen sowie deren jeweiligen physiologischen Status in einer wässrigen Probe (Grafik 1).



Grafik 1. Schematische Darstellung des CellFacts II-Prinzips. Partikel (oder Zellen) in einer konduktiven Matrix passieren die Messzelle mit einem Durchmesser von 30 µm.

Der Erfolg und Gehalt der Aussagen liegt auf der Hand. Optische Methoden (Light Scattering) haben den Nachteil der ungenauen Durchmesser- bzw. Volumenmessung zur Erfassung der Grösse einer Zelle. Dieses liegt primär am Brechungsindex unterschiedlicher Zellen im Verhältnis zum umgebenden Medium. Die chemische Struktur von Zellen ist unterschiedlich im Aufbau an der Hülle (z.B. Membranproteine der Zellwand/Cytoplasma). Dieses hat zur Folge, dass der Brechungsindex unterschiedlich ist und im umgebenden, flüssigen Medium, eine kleine Zelle grösser erscheinen lässt und umgekehrt.

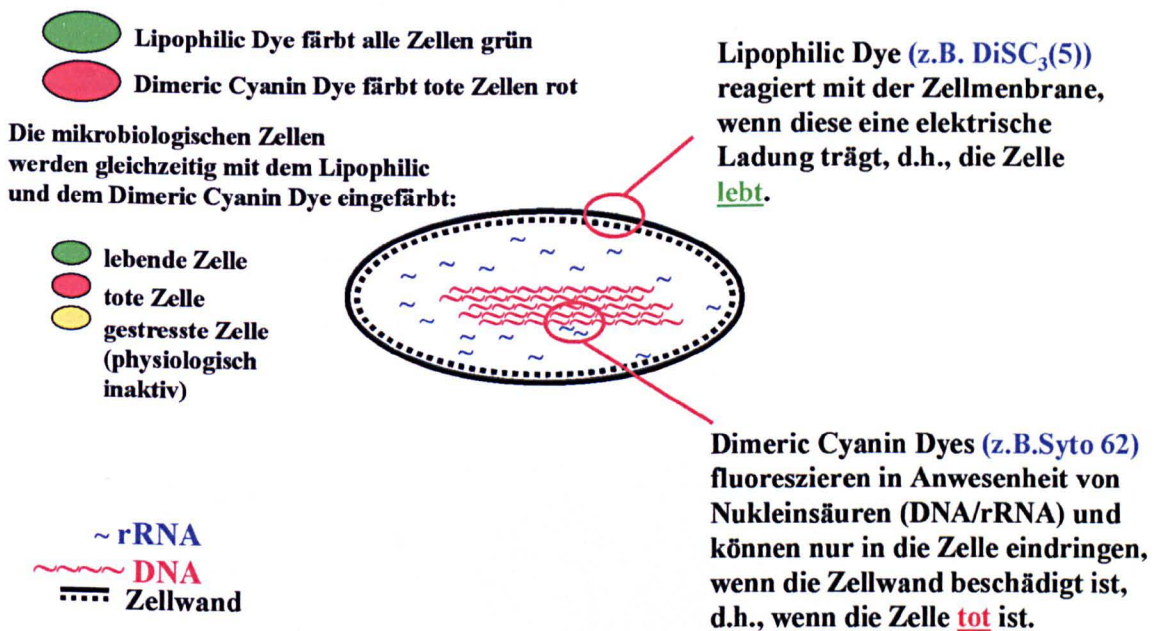
Hier bringt die elektrische Impedanz entscheidenden Vorteile. Bei dieser Messmethode ist das Verhältnis von Teilchengrösse (Zellgrösse) zum Durchmesser der Messpore entscheidend für die Auflösung mit dem gemessenen Volumen über der Zeit. Die elektrische Leitfähigkeit von Zellen ist tiefer als die der Elektrolytlösung, so dass beim Durchtritt einer Zelle durch die Messpore eine



niedrige Leitfähigkeit gemessen wird, wobei das Volumen der Zelle der Pulshöhe (Peak) entspricht. So wird beides gemessen: die Grösse und die Anzahl der Zellen zum Zeitpunkt X.

## Fluoreszenz zur Charakterisierung des physiologischen Status einer Zelle

Um den physiologischen Status einer Zelle zu definieren, würde man bei der elektrischen Methode (Impedanz) mehrere Messungen brauchen, denn diese bestimmt wohl die Grösse und die Anzahl der Zellen, unterscheidet aber nicht ob diese tot, lebend oder physiologisch inaktiv sind. Addiert man aber durch einen weiteren Detektor ein zusätzliches Signal, z.B. Fluoreszenz, können mit einer Messung Aussagen über den physiologischen Status der Zellen gemacht werden (Grafik 2, Bild 1).



Grafik 2 zeigt, wie durch das Einfärben der Zellen verschiedene Fluoreszenz-Intensitäten in Abhängigkeit vom physiologischen Status gemessen werden können. Die Signale sind zudem spezifisch auf die Zellgrösse (z.B. Kokken, Stäbchen oder grössere Zellen wie Hefen).



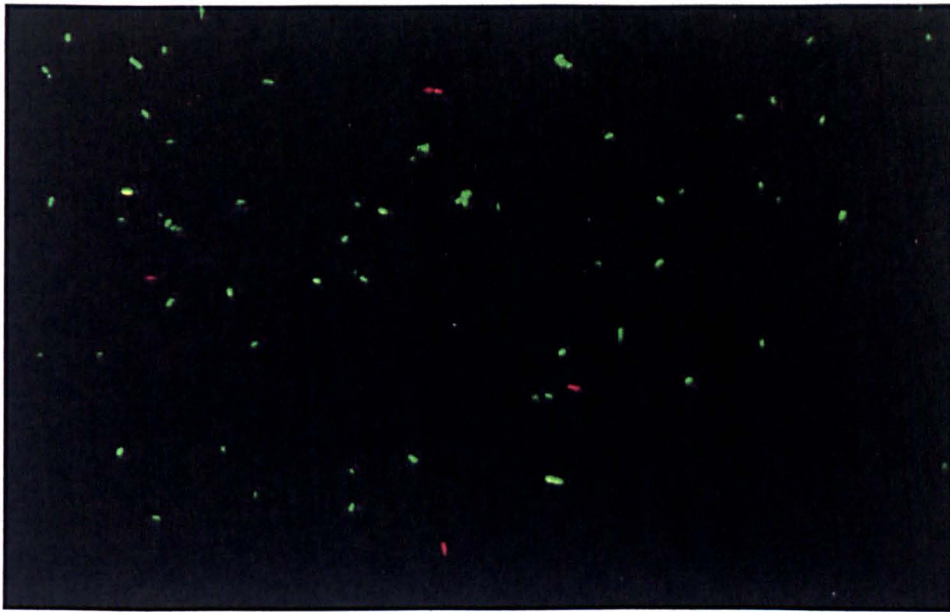
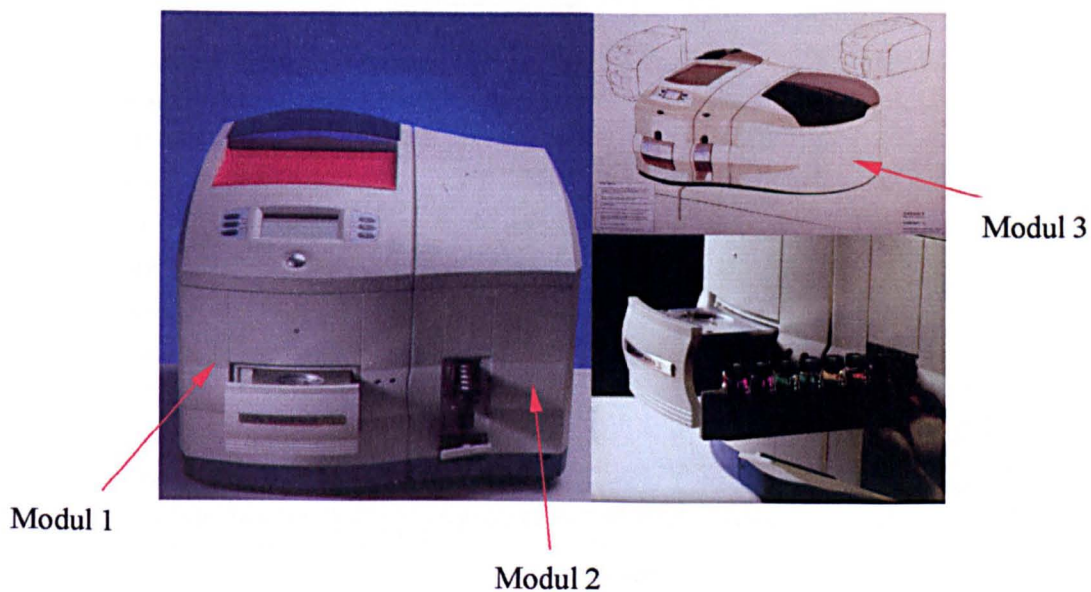


Bild 1. Aus einer Dispersionsfarbe isolierte Zellen nach dem Anfärben, betrachtet unter dem Phasenkontrastmikroskop mit Fluoreszenz. Die grünen Zellen sind physiologisch aktiv, die roten hingegen tot.

## CellFacts II und seine Möglichkeiten

Das Gerät setzt sich aus zwei Modulen zusammen und kann für grössere Probemengen durch einen automatischen Probenwechsler (Modul 3) ergänzt werden (Bild 2).



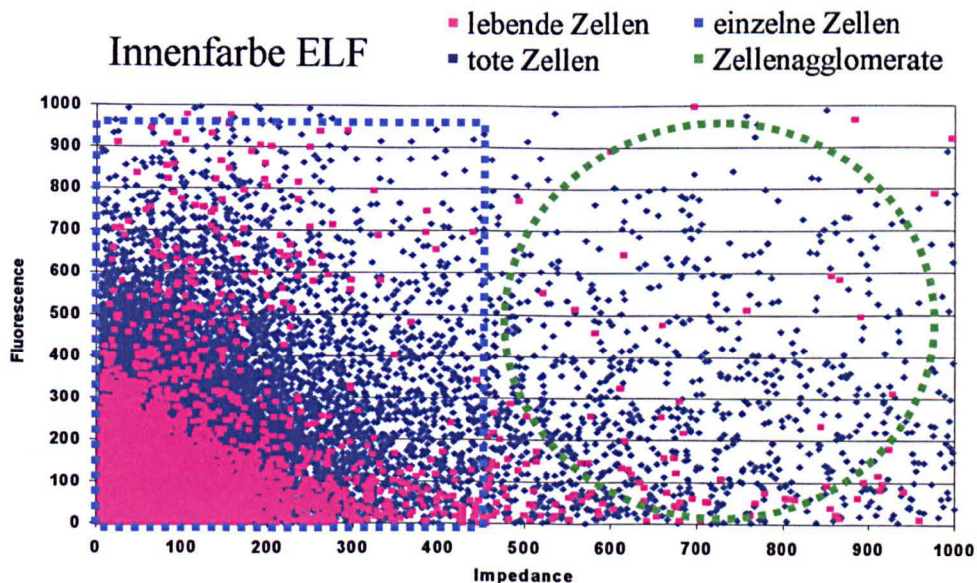
## Bild 2. CellFacts II Module:

- Modul 1** Analysen-Modul, Integration der Impedanz- und der Fluoreszenzsignale sowie Messung der individuellen Partikel oder Zellen.
- Modul 2** Reagenz Additions-Modul. Mit diesem Modul können bis zu sieben Reagenzien (Fluoreszenzmarker, Biozide, etc.) zugefügt werden. Weiter besteht die Möglichkeit dieses als kleinen Probewechsler einzusetzen.
- Modul 3** (Hier als original Konzeptzeichnung gezeigt) ist ein automatischer Probewechsler welcher bis zu 150 Proben bearbeiten kann. Weiter besteht die Möglichkeit die Proben mittels Barcode einzulesen und zu definieren.

## Applikation im Bereich der mikrobiologischen Qualitätssicherung

Für den Einsatz von CellFacts II gibt es gute Gründe. Mit nur einer Messung lassen sich unterschiedliche Keime bis zu einer Nachweisgrenze von 100 Keimen/ml exakt bestimmen; differenziert in lebende bzw. tote Zellen und das alles in Echtzeit: Während man misst, erscheint das Ergebnis.

Diese Palette an Leistungsmerkmalen eröffnet CellFacts II ein breites Anwendungsspektrum in allen Bereichen der Mikrobiologie, aber auch in klassischen Industrien wie der Pigment-, Farben- & Lack oder Papierindustrie, in denen mikrobiologische Fragen eine Rolle spielen. CellFacts II ist



verwendbar für eine gemischte Flora wie für pathogene Keime und kompatibel mit existierenden Techniken wie Fluoreszenzmarkern, Antikörper-Labeling und Immunologie.

Grafik 3. Um das Verhältnis von der physiologisch aktiven (lebend) Keimzahl mit der toten Keimzahl in dieser Innendispersionsfarbe zu verdeutlichen, wurden die beiden Resultate in der Grafik überlagert. Diese Technik kann auch genutzt werden, um die Wirksamkeit eines Biozides zu veranschaulichen.

Weiter ist der Unterschied zwischen einzelnen Zellen und Agglomeraten von Zellen deutlich sichtbar.

Durch den Einsatz dieser neuen Technik wird auch in der Farb- & Lackindustrie ein neuer Standard geschaffen. Mikrobiologische Qualitätsprüfungen von wässrigen Farbsystemen, Rohstoffen und Additiven wie auch die Prüfung der bakteriziden Wirkung von Konservierungsmitteln stellen nur einen Teil der Fülle an Informationen dar, welche dem Anwender innerhalb weniger Minuten zur Verfügung stehen. Das macht CellFacts II überall dort zum idealen Messgerät, wo es um den schnellen Nachweis von Keimen geht.

# A New Analytical Method in Microbiology

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**Summary:** A new analytical method is described that integrates electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics of individual cells in a conductive fluid. The technique has been optimised to detect and enumerate viable and non-viable cells in fluid samples with varied particulate content, i.e. total viable counts, together with discrimination of the physiological status of the individual cells, and has been developed into a measuring device.

The study shows the analysis of the physiological state of individual cells in a population, effectively in real-time, enabling the rapid determination of the effect of antimicrobial agents on these cells. Applications are described where the methodology is used for the rapid identification and optimisation of antimicrobial agents in aqueous paint systems. This procedure is of ever-increasing importance since antimicrobial agents have to meet the new EU Biocidal Product Directive (BPD), and paint systems will be evaluated according to the directive RAL UZ12a (Blauer Engel).

## Introduction

To meet today's high requirements with respect to microbiological management in paint products, it is essential that the following issues be taken into account.

- regular good house-keeping procedures
- the use of efficient and approved preservatives and disinfectants
- the ability to perform rapid monitoring of the system.

The mutual inter-dependence of these points is obvious. A fast monitoring system can effectively enable the quantity of disinfectant and preservative in use to be reduced due to fast reaction potential, as well as permitting any good house-keeping initiatives to be optimised.

Traditional microbiological analyses for the determination of the presence of low levels of contaminating microorganisms, such as the well-known Plate Count, are time consuming, often taking from 2 to 3 days to complete. In addition, there are various other factors to be considered, such as, for example, the type of culture medium to be used, the effect of the partial pressure of oxygen (aerobic / anaerobic), various selectivity mechanisms, pH value and many more.<sup>[1-2]</sup>

A rapid method that could provide an accurate assessment of the number of microorganisms

present would allow positive release of the paint product to the marketplace in the knowledge that it was free from effective contamination.

Such a new method, described here, integrates electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics (e.g. viability, physiological status, speciation) of individual particles or cells in a conducting liquid. The instrument has been optimised and sample preparation methodology developed to detect and enumerate viable and non-viable cells in fluid samples with varied particulate content, i.e. total viable counts, together with discrimination of the physiological status of the individual cells (also discrimination between cells of different sizes, e.g. between yeast and bacteria).

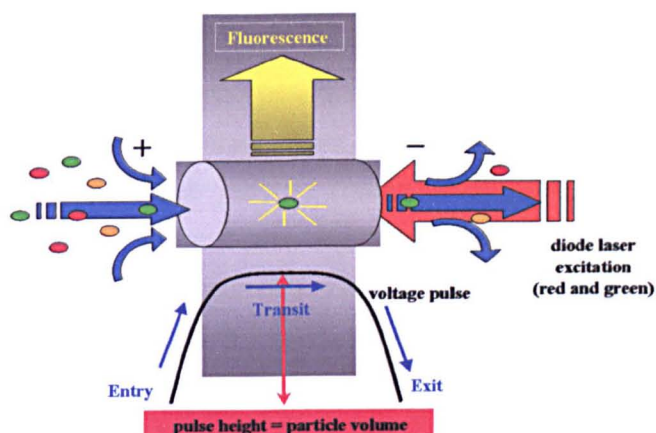
The following study shows the analysis of the physiological state of individual cells in a population, effectively in real-time, enabling the rapid determination of the effect of antimicrobial agents on these cells in pigmented systems such as those found in paints. Since antimicrobial agents have to meet the new Biocidal Product Directive (BPD), and paint systems will be evaluated according to the directive RAL UZ12a (Blauer Engel) a concerted effort to promote a proactive microbiological control protocol becomes ever more important.

### **The Operating Principle**

The new analytical instrument uses patented technology integrating electrical flow impedance and fluorescence to determine the number, size and fluorescence characteristics of microbiological particulate material. This is used to determine the viability, physiological status and speciation of individual particles or cells in a conducting liquid (Figure 1).

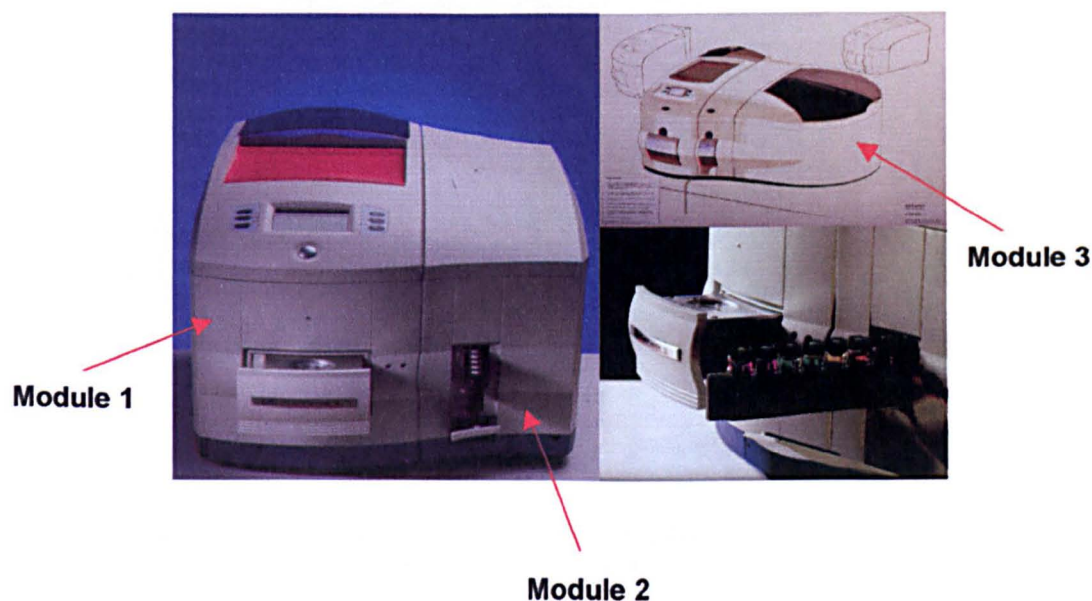
Two diode lasers (red and green) are focussed onto the orifice. As a particle (or cell) enters the orifice it displaces its own volume of electrolyte and this generates a voltage pulse, the magnitude of which is directly proportional to the volume of the particle. This signal undergoes detailed analysis via the specialised software and is used to generate information on particle (or cell) size and the numbers within a given population. It may also be used to trigger fluorescence characterisation of the particle as it transits the orifice by integration of the impedance and fluorescence data. A key feature is the orifice blocking detection system which ensures the instrument operates irrespective of the size heterogeneity of the particles existing on the sample side of the orifice, resulting in an extremely effective anti-blocking system.<sup>[3]</sup>





**Figure 1.** Schematic representation of the principle.<sup>[4]</sup> Particles (or cells), in a conducting liquid, pass through a 30  $\mu\text{m}$  diameter orifice which has been laser etched into a 80  $\mu\text{m}$  sapphire disk.

The instrument has a modular configuration (Figure 2) to meet the requirements of the broad spectrum of applicable market sectors.

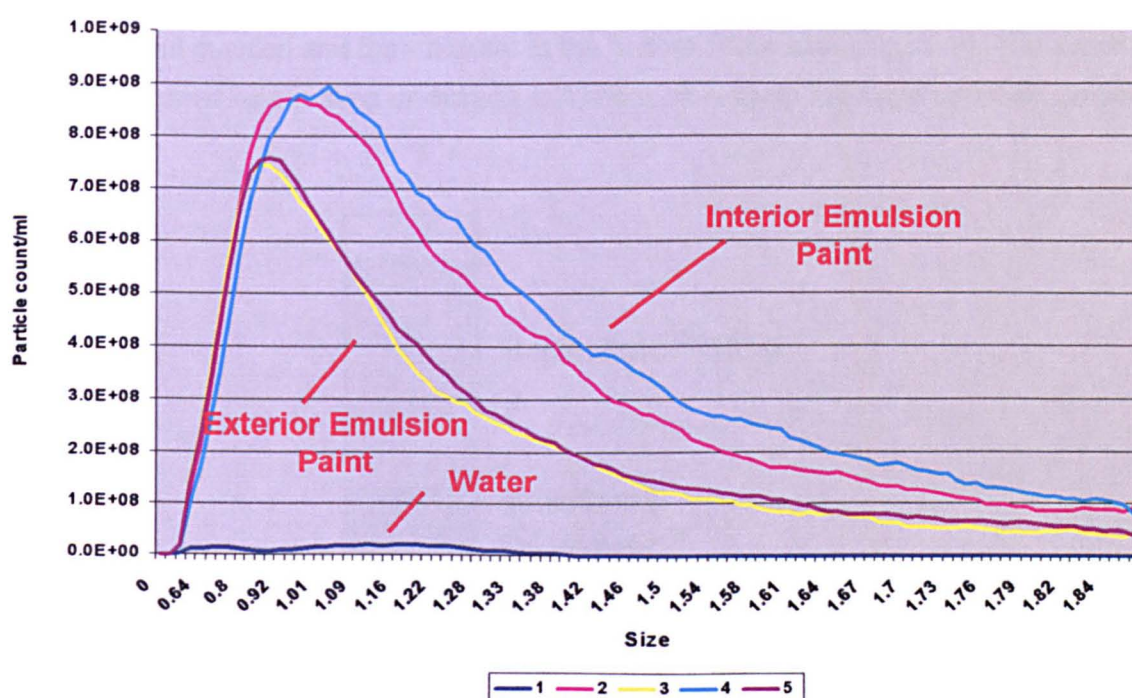


**Figure 2.** Instrument Modules. The modules consist of:

- Module 1** Analysis module, integrating both impedance and fluorescence measurements of individual particles or cells.
- Module 2** Reagent addition module. This unit facilitates the addition of up to seven different reagents enabling multiple, complex, automated analyses to be undertaken for each sample.
- Module 3** Shown here in the original as yet concept drawing, is an automated sample-handling unit with sample mixing and bar code facilities.

## Sample Preparation

A patented sample preparation technique is followed to allow the instrument to be used with high solids suspensions containing particulate fillers, extenders and pigments, such as paints, by extracting preferentially the microbial material. Clearly, the challenge in preparing the sample is the separation of the microorganisms from the matrix. To be able to determine just a few bacterial cells in a matrix with a high number of other "background" particles of occurrence ratio up to  $1.0 \times 10^{10}$ , separation through centrifugation is essential. Figure 3 shows a typical situation when the total number of particles is present in a sample. Clearly, the bacteria are effectively lost in such a particle size distribution (size shown in  $\mu\text{m}$ ).



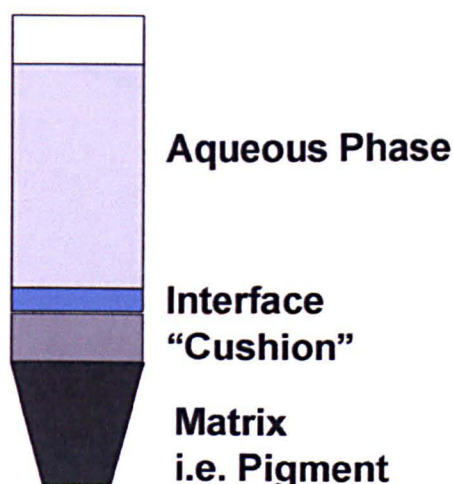
**Figure 3.** Detection of cells in a matrix with a high number of particles is impossible. However, the low concentration of microbial material in water is detectable by means of electrical impedance.

Initial sample preparation procedures consisted of dilution of the aqueous paint systems followed by direct centrifugation. The supernatant was then separated and used for analysis. The recovery of bacteria from the samples was typically  $\sim 10\%$  of those suspected to be present (determined on PCA agar plates from pre- and post-spin samples), although this recovery can range from  $< 1\%$  to complete recovery depending on the systems involved - depending especially on the types of dispersing technologies in use.



The newly developed sample preparation method, mentioned at the beginning of this section, which was in another form originally used successfully to concentrate and isolate microbes from soil <sup>[5]</sup>, relies upon a density gradient cushion to separate bacteria from the matrix during centrifugation. This technique essentially entails centrifugation of an aqueous paint system in the presence of a disruption buffer mixture which is pipetted onto the top of a layer of a density gradient cushion material (1.3 g/ml).

Following centrifugation, the supernatant, which contains an upper aqueous phase and the "density gradient" solution, is collected. Since bacteria are resolved at the interface between the two layers they will be present in the supernatant, while pigment particles can pass through the density gradient cushion and form a pellet at the bottom of the tube (Figure 4). The supernatant is easily separated by pipetting or decanting it into a new sterile tube and can then be used for the analysis.

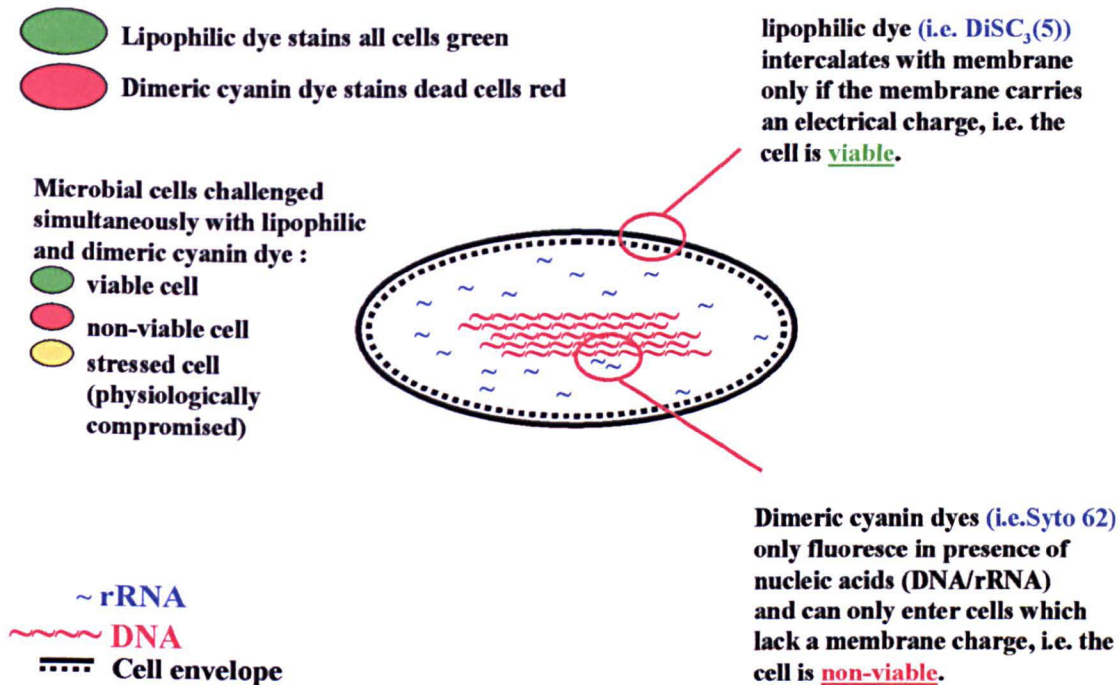


**Figure 4.** Separation of bacteria from the matrix by using a density gradient cushion.

In practice, the sample preparation can be semi-automated to minimise operator intervention.

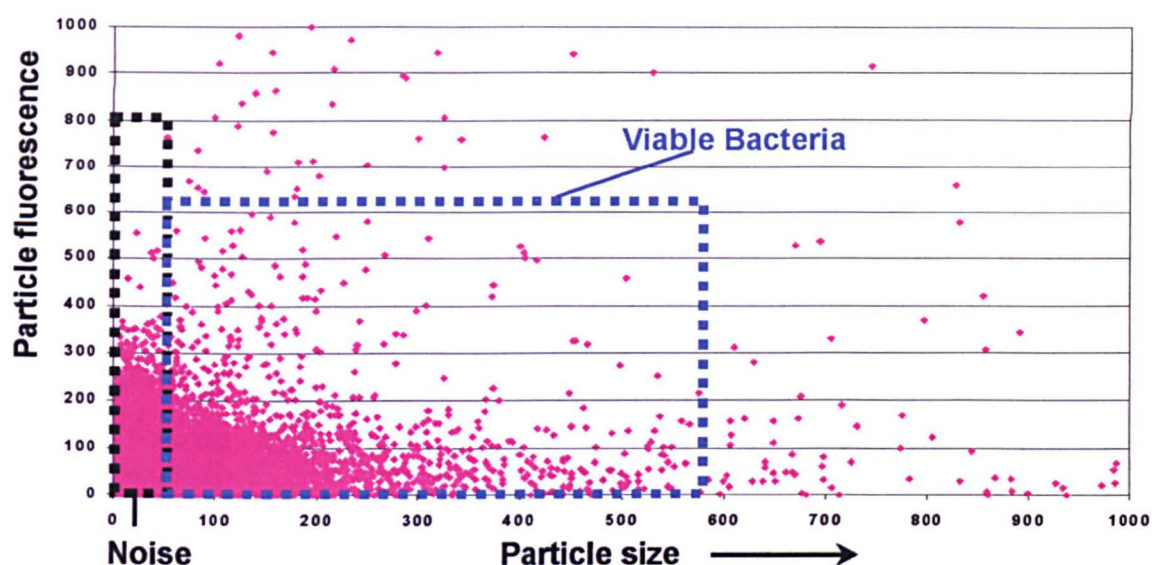
## Fluorescence: Characterising the Physiological Status of Cells

Following the sample preparation procedure, a protocol is applied by which, using minimal staining with dimeric cyanine nucleic acid dyes and lipophilic cationic dyes (membrane potential dyes which act as viability indication dyes), it is possible to characterise the physiological status of individual cells, as described in Figure 5 <sup>[6-7]</sup>.



**Figure 5.** The selective dye absorption provides discrimination depending on physiological status.

The signal from the instrument can be displayed as the particle fluorescence distributed over the measured range of particle size, as shown in Figure 6, in which the total viable count is detected within the particle size range typically associated with the microbial material.



**Figure 6.** This graph clearly shows the area of the viable bacteria defined by fluorescence (MESF = molecular equivalent of soluble fluorochromes) and particle size (channels). The total viable count detected in the sample shown (Interior Emulsion Paint 4B - data below in Table 1) was  $3.5 \times 10^3$  cfu/ml.

## RESULTS

A study was carried out comparing the new analytical method, involving the specialised sample preparation described above, with traditional techniques, such as Plate Count, for evaluating the potential application in aqueous paint systems. The preservative properties of commercially available paint systems were also tested and different antimicrobial agents were tested against one another.

### Total Viable Count

On comparing the total viable count of all the samples tested (Table 1) it becomes clear that the results using the new technology tend to be somewhat higher than those of the traditional plate count method. This result is in fact to be expected, and is explicable due to the specific test conditions chosen for the traditional culture evaluation (nutrient, pH, temperature). In effect, this shows that not all the viable cells are detected by the plate count method! These cells are successfully detected by the new method, and, what is new, in real time<sup>[8]</sup>.

It is indeed possible that cells present in a system which traditional methods cannot detect can have a negative impact on the properties of the product.



**Table 1. Total Viable Count of 16 Aqueous Paint Systems**

Sample	PCA <sup>a)</sup> (cfu/ml) <sup>b)</sup>	NAM <sup>c)</sup> (cfu/ml)
Interior Emulsion Paint 1A	< 100	< 100
Interior Emulsion Paint 1B	< 100	< 100
Exterior Emulsion Paint 1C	< 100	< 100
Exterior Emulsion Paint 1D	< 100	< 100
Interior Emulsion Paint 2	< 100	$3.0 \times 10^2$
Exterior Emulsion Paint 2	< 100	< 100
Interior Emulsion Paint 3	$2.0 \times 10^2$	$1.3 \times 10^3$
Exterior Emulsion Paint 3	< 100	< 100
Exterior Emulsion Paint 4A	$1.0 \times 10^2$	$7.8 \times 10^3$
Interior Emulsion Paint 4B	< 100	$3.5 \times 10^3$
Exterior Emulsion Paint 4C	< 100	< 100
Interior Emulsion Paint 4D	< 100	< 100
Interior Emulsion Paint 5A (without biocide)	< 100	$1.8 \times 10^3$
Exterior Emulsion Paint 5B (without biocide)	$7.0 \times 10^2$	$6.7 \times 10^3$
Interior Emulsion Paint 5C (without biocide)	$5.0 \times 10^4$	$9.4 \times 10^4$
Exterior Emulsion Paint 5D (without biocide)	$8.0 \times 10^4$	$4.9 \times 10^5$

a) PCA = Plate Count Agar

b) cfu = cell forming unit

c) NAM = New Analytical Method

### Stability of Preservation

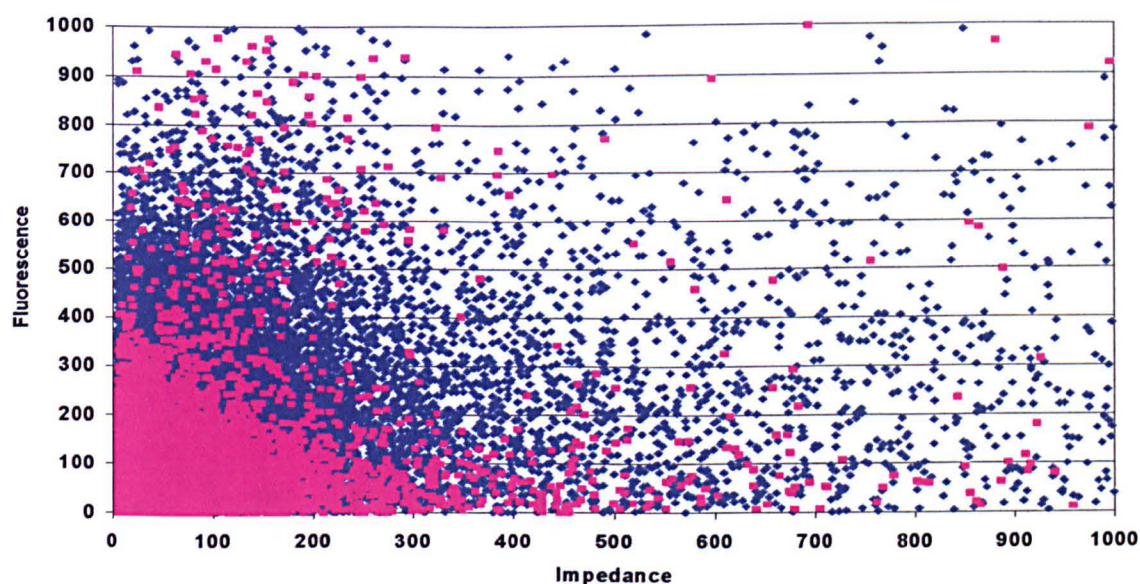
The samples in the series 1 - 4 were subsequently tested to establish their existing level of preservative properties. For this purpose, the samples were inoculated with a quantity of  $5.0 \times 10^4$  bacteria per ml isolated from typical aqueous paint systems. After the subsequent storage of the samples for 24 hours at 30°C, the total viable count was determined by means of the new analytical method. Additional inoculations were made until the total viable count of each sample was  $> 10^4$  cfu/ml (Table 2). The number of possible additional inoculations without viable contamination, therefore, gives an indication of the quality of the preservative action present in the product.

The results show that significant differences can be found between the various samples analysed. In this study the paints from series 1 were identified as having the best preservative properties.

**Table 2. Stability of Preservation of 12 Aqueous Paint Systems**

Sample	TVC <sup>a)</sup> before Inoculation	Number of Inoculations
Interior Emulsion Paint 1A	< 100	4
Interior Emulsion Paint 1B	< 100	4
Exterior Emulsion Paint 1C	< 100	3
Exterior Emulsion Paint 1D	< 100	4
Interior Emulsion Paint 2	$3.0 \times 10^2$	1
Exterior Emulsion Paint 2	< 100	2
Interior Emulsion Paint 3	$1.3 \times 10^3$	1
Exterior Emulsion Paint 3	< 100	2
Exterior Emulsion Paint 4A	$7.8 \times 10^3$	1
Interior Emulsion Paint 4B	$3.5 \times 10^3$	1
Exterior Emulsion Paint 4C	< 100	3
Interior Emulsion Paint 4D	< 100	2

a) TVC = total viable count



**Figure 7.** This graph shows the Interior Emulsion Paint, taken from series 3, before (violet) and after (blue) the inoculation amounting to approx.  $5.0 \times 10^4$  cells/ml. The initial total viable count of a test paint (series 3) was measured as  $1.3 \times 10^3$  cfu/ml. After inoculation and incubation for 24 hours at 30°C, the total viable count rose to  $2.6 \times 10^6$  cfu/ml. The residue of biocide in the sample was not sufficient to prevent the further contamination developing into viable microbial activity (fluorescence in MESF = molecular equivalent of soluble fluorochromes, impedance in channels).

### Performance of Different Antimicrobial Agents in Aqueous Paint Systems

In a further test the bactericidal properties of different active agents were compared by means of the new analytical method. The following substances were used in this study:

- 2-methyl-2H-isothiazole-3-one (MIT) / 5-chloro-2-methyl-2H-isothiazoline-3-one (CMIT) (CMIT:MIT = 3:1)
- 1,2-benzisothiazole-3(2H)-one (BIT)
- o-phenylphenol (OPP)
- 2-bromo-2-nitro-propane-1,3-diol (bronopol)

For this purpose, sterile aqueous paint samples were mixed with controlled quantities of the active agents, which are usually added during production, and stored for 24 hours at 30°C to achieve thorough mixing and to develop an equilibrium distribution of the active agent. The samples were then inoculated with bacteria isolated from typical aqueous paint samples. Measurements of the viable count were made after 15 min., 30 min., 60 min., 2 hours, 4 hours, 6 hours and 8 hours. Both the decrease of the viable bacteria due to the action of the biocide, as well as the increase of the resulting dead bacteria were evaluated by means of the dye fluorescence protocol.

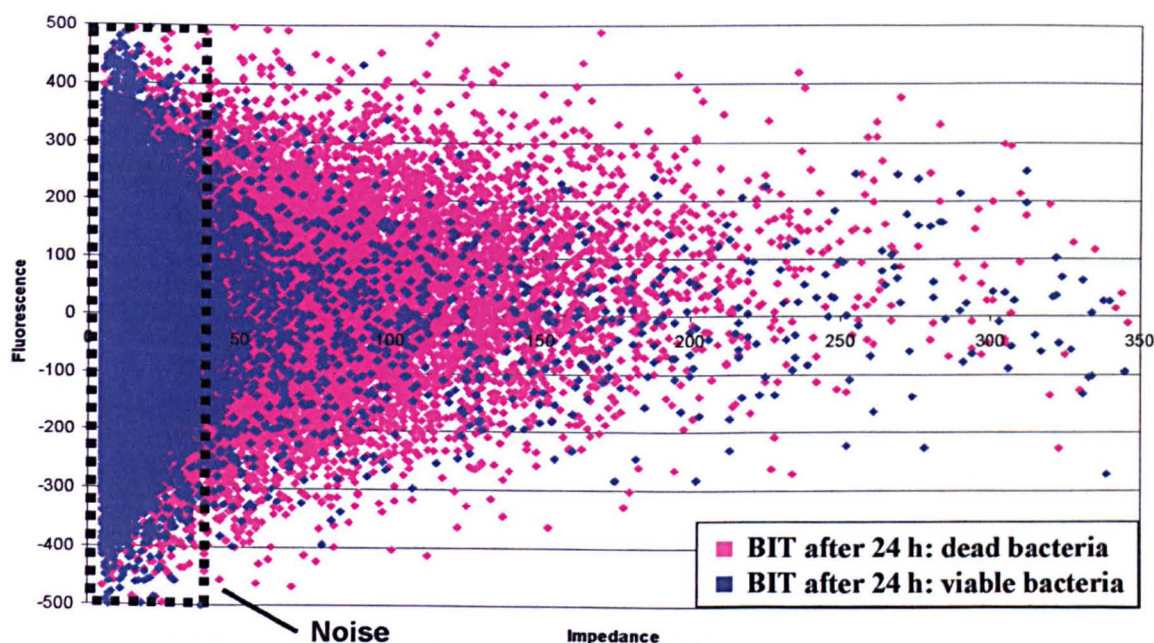
**Table 3. Performance of Different Antimicrobial Agents in Aqueous Paint Systems**

Biocide	Time	% of cells detected as viable	% of cells detected as dead
MIT/CMIT	0 min	100	0
MIT/CMIT	15 min	97	3
MIT/CMIT	30 min	72	28
MIT/CMIT	60 min	16	84
MIT/CMIT	2 h	0	100
BIT	0 min	100	0
BIT	15 min	100	0
BIT	30 min	87	13
BIT	60 min	69	31
BIT	2 h	47	53
BIT	4 h	38	62
BIT	6 h	35	65
BIT	8 h	32	68
BIT <sup>a)</sup>	24 h	32	68

OPP	0 min	100	0
OPP	15 min	37	63
OPP	30 min	0	100
Bronopol	0 min	100	0
Bronopol	15 min	98	2
Bronopol	30 min	82	18
Bronopol	60 min	65	35
Bronopol	2 h	30	70
Bronopol	4 h	9	91
Bronopol	6 h	0	100

a) To detect any possible deficiency in the action of the biocide, the samples were again measured 24 hours later.

Table 3 shows that BIT has a deficiency in this application in its action against some of the bacteria used in this study, which incidentally would not necessarily be recognised by the traditional methods due to their culture selectivity. Furthermore, it could be shown that the fastest bactericidal action under the test conditions was achieved with OPP, whereas with Bronopol developed its bactericidal effect only after 6 hours.



**Figure 8.** In this graph the detected deficiency in the preservative action of BIT is illustrated. After 24 hours reaction time there are still viable bacteria. BIT is well-known for its deficiency in the action against some species of *Pseudomonas* <sup>[9]</sup> (fluorescence in MESF = molecular equivalent of soluble fluorochromes, impedance in channels).

## Conclusion

A new analytical method has been presented and shown to have a ready application-oriented potential for both analytical flexibility and sensitivity. The methodology which allows the testing of suspensions with high solids content of pigment materials provides a special applicability to paints. Its use involves minimal operator intervention outside the sample preparation procedures which themselves can be semi-automated.

The sensitivity and specificity of each analytical mode is given by the ability to determine the particle heterogeneity of the sample and the staining characteristics of the microbial population, which in turn reflects the physiological status of the individual cells. Also, discrimination between cells of different sizes, e.g. between yeast and bacteria, is possible.

The resulting analysis of the physiological state of individual cells in a population, effectively in real-time, enables the rapid determination of the effect of antimicrobial agents on these cells, i.e. rapid determination and optimisation of antimicrobial agents.

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